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Science and Technology of Archaeological Research

Research Paper

Calculating a statistically robust $\delta^{13}C$ and $\delta^{15}N$ offset for charred cereal and pulse seeds

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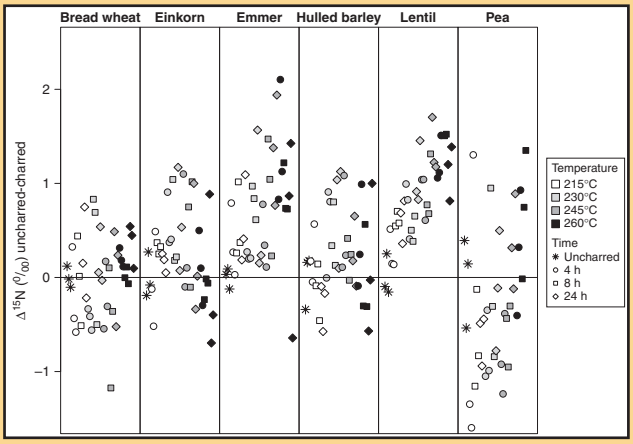
Abstract This study measured $\delta^{13}C$ and $\delta^{15}N$ of fresh and charred seeds from six different taxa of cereals and pulses. For each taxon, 12 different batches of seeds, all from the same growing context or field, were charred for 4, 8, or 24 hours at 215, 230, 245, or 260°C, with the thirteenth batch left uncharred. The least charred samples showed no average offset compared to their uncharred counterparts, but among the charred seeds, every 15°C increase resulted in an increase in $\delta^{15}N$ by 0.12‰, while increasing the duration of charring by 4 hours resulted in an average increase of 0.04‰. Differences in $\delta^{13}C$ among the charred samples were only minimally dependent on time (+0.016‰/4 hours duration), but not temperature. The average offset for all batches and all taxa was minimal for $\delta^{13}C$, at +0.11‰ (95% confidence interval (CI) 0.003–0.22‰). The average offset for $\delta^{15}N$ of 0.31‰ (95% CI 0.05–0.57‰) is smaller than the conservative estimate of 1‰ used in previous studies. We demonstrate that using this new, smaller charring offset for $\delta^{15}N$ makes a significant difference in paleodietary models, one that further emphasizes the potentially high $\delta^{15}N$ of manured crops relative to animal forage.



Statement of significance Archaeologists and scientists measure carbon and nitrogen isotope ratios in ancient crop remains that have been preserved by charring to understand how crops were grown in the past. The charring process preserves the seed's physical form but results in a slight alteration to the carbon and nitrogen isotope ratios. Our study systematically measured this offset within a window of charring temperatures and times: a window in which the seeds and grains have been completely chemically converted into a stable form, but where this process has been gentle enough that the physical form of the seed has not been distorted. From this study, we understand more precisely the size of the small adjustment we need to make to our measurements of ancient charred crop remains so that we can work back to the original values that reflect the conditions in which these crops were once grown.

Keywords Archaeobotany, Charring, Carbonization, Carbon isotope analysis, Nitrogen isotope analysis, Stable isotope analysis

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Data availability The authors confirm that all data underlying the findings are fully available without restriction. All relevant data are contained within the paper.

Introduction

The ability to measure $\delta^{13}C$ and $\delta^{15}N$ of archaeobotanical grains and seeds, and to relate these values to those of living plants, has opened up a new field of isotopic research in archaeology. It allows the reconstruction of crop management practices, such as manuring and irrigation, and environmental conditions, such as aridity (Aguilera *et al.* 2008; Araus *et al.* 1997a, 1997b; Bogaard *et al.* 2013; Ferrio *et al.* 2005; Fiorentino *et al.* 2008, 2012; Heaton *et al.* 2009; Masi *et al.* 2014; Riehl 2008; Riehl, Bryson and Pustovoytov 2008; Vaiglova *et al.* 2014; Wallace *et al.* 2013). Direct measurement of cereal $\delta^{15}N$ also provides a significant improvement to isotopic paleodietary reconstructions (Fraser *et al.* 2013a; Styring *et al.* 2015). The successful application of this technique

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depends on being able to relate the measured $\delta^{13}C$ and $\delta^{15}N$ in the preserved archaeobotanical sample to its original value.

Grains have two main components: the protein- and lipid-rich embryo (reproductive component) and the starch-rich endosperm, both surrounded by protective outer layers (nucellus, testa, and other epidermal structures). The starchy endosperm typically accounts for 60–85‰ of the mass of the grain, and is made up of a mass of starch granules embedded in a protein matrix, surrounded by aleurone cells that have higher concentrations of lipids and proteins (Evers and Millar 2002). The structure of pulses, such as peas and lentils, is similar, except that they lack the starchy endosperm and rely solely on the starch component of the embryo's cotyledons for energy, with the result that the overall crude protein content (*N*×6.25) of pulses (20–30%) is higher than that of cereals (10–14%) (Wang and Daun 2003; Metayer, Grosjean and Castaing 1993).

Charred cereal grains and pulses survive burial for thousands of years because exposure to heat initiates Maillard reactions, transforming the seeds' starches and amino acids into high-molecular weight melanoidins, whose complex structure is resistant to microbial attack (Braadbaart *et al.* 2004a, 2004b, 2004c; Styring *et al.* 2013). To reconstruct the original carbon (C) and nitrogen (N) isotopic ratios, it is essential to know what effect charring may have on the bulk seed $\delta^{13}C$ and $\delta^{15}N$, especially if fractions of differing isotopic ratio may be preferentially lost or retained.

Charring

Charring results in visible alterations to seeds (cereal grains and legume seeds), changing the colour from pale brown to black and distorting the morphology. Terms such as 'charring', 'carbonization', or 'charcoalification' all describe processes along a 'combustion continuum' where different chemical changes occur with increasing temperature, until ultimately only pure graphite remains (Hedges *et al.* 2000; Masiello 2004). Charred material becomes progressively C rich, with decreasing %H and %O, coincident with the formation of condensed aromatic structures (Bird and Ascough 2012). Differences in the bulk $\delta^{13}C$ and $\delta^{15}N$ ratio of charred seeds compared to uncharred seeds, which we refer to as 'offsets', may occur due to kinetic effects during chemical reactions or due to the preferential loss of biochemical components with substantially different isotopic ratios.

In wood charcoal, the different types of compounds formed due to charring are largely temperature dependent. Lipids and other volatiles such as water are lost at relatively low temperatures (<150°C) (Czimczik *et al.* 2002), and since lipids have relatively negative $\delta^{13}C$ compared to the bulk material (Benner *et al.* 1987), the loss of lipid will increase the apparent $\delta^{13}C$ slightly (Czimczik *et al.* 2002). At slightly higher temperatures (between 220 and 300°C), cellulose and other starches break down, although they are initially retained as charcoal in the matrix of the plant before beginning to volatilize beyond 300°C (Ascough *et al.* 2008; Hakkou *et al.* 2006; Jones, Scott and Mattey 1993; Qian et al. 1992; Williams and Besler 1996; Xiao, Sun and Sun 2001). Since cellulose is isotopically heavy relative to bulk composition (Benner *et al.* 1987), loss of cellulose C beyond 300°C will decrease bulk $\delta^{13}C$ further (Czimczik *et al.* 2002). Charcoal that is formed without the complete conversion of starch to aromatic C will likely not survive if exposed to an oxidizing environment (Ascough *et al.* 2010).

While much of the information about cellulose and lipid breakdown can be applied to seeds, the significantly higher N content of seeds means that other reactions will occur. Under heat treatment, amino acids and starch will undergo Maillard reactions, forming heterocyclic aromatic, cross-linked products and high-molecular weight melanoidins, which are resistant to microbial attack (Knicker *et al.* 1996; Silván *et al.* 2006; Styring *et al.* 2013). This process produces the black colour change, which is characteristic of archaeological charred material (Boardman and Jones 1990). Similar to wood charcoal, complete or nearly complete conversion of starch and proteins into stable aromatic compounds is a necessary requirement for preservation (Ascough *et al.* 2010; Braadbaart *et al.* 2004b, 2004c).

This breakdown of starch and protein is linked to overall mass loss. Braadbaart et al. (2004b, 2004c) found that peas and emmer wheat heated for 1–2 hours and 2–2.5 hours, respectively, showed a significant increase in mass loss between 220°C (~18% mass loss) and 250°C (~40%), mirroring the mass loss pattern of pure microcrystalline cellulose at similar temperatures (Pastorova, Arisz and Boon 1993). For wheat grains and peas, a greater than 50% mass loss coincided with the complete conversion of starch and proteins, which occurred with heating at 310°C or beyond for 2–2.5 hours (Braadbaart *et al.* 2004a, 2004b, 2004c; Braadbaart and van Bergen 2005).

Although such conditions adequately replicate the blackening and starch-loss of archaeobotanical grain, they result in significant morphological distortions, including swelling, protrusions, and distortions in emmer and free-threshing wheat grain, while the cotyledons of peas start to crack at temperatures greater than 310°C (Braadbaart 2008; Braadbaart and van Bergen 2005; Braadbaart *et al.* 2004b, 2004c; Märkle and Rösch 2008). Since the focus of archaeobotanical research is on those minimally distorted grains that are most identifiable, recent effort has focused on the effects of less intense heat exposure (<250°C), as part of a 'smouldering building' model of charring (Charles, Forster and Jones Submitted).

Experimental charring of emmer and einkorn for 2–24 hours at temperatures ranging from 200 to 260°C and analysis of cross-sections under conventional and scanning electron microscopy reveal that grains charred under such conditions exhibit loss of starch granules but lack significant morphological distortions (class 1 or 2 after Hubbard and al Azm 1990), in a way that mimics the structure observed in preserved

archaeobotanical grain (Charles *et al.* Submitted). Cereals and pulses charred for longer durations and at lower temperatures (230°C for 24 hours) produce similar mass loss patterns to those achieved in hotter, shorter duration experiments, of between 45 and 55% (Fraser *et al.* 2013b). Under such temperatures, almost all the starch and amino acids were found to have been converted in the actual charring event, and it is likely that the Maillard reactions initiated by the charring would continue at room temperature, albeit at a lower rate (Benzing-Purdie, Ripmeester and Ratcliffe 1985; Styring *et al.* 2013). Long-term burial and contamination experiments also suggest that grains, having been charred, will preserve their isotopic composition following burial (Fraser *et al.* 2013b; Styring *et al.* 2013).

Previous isotopic study of changes in $\delta^{13}C$ and $\delta^{15}N$ due to charring

The feasibility of $\delta^{13}C$ and $\delta^{15}N$ measurement of charred grain was initially proposed by DeNiro and Hastorf (1985) who found that charred seed isotopic ratios were strongly related to their uncharred values. Most studies of $\delta^{13}C$ find minimal differences due to charring in cereals and pulses (Araus *et al.* 1997b; Fiorentino *et al.* 2012; Fraser *et al.* 2013b; Marino and DeNiro 1987), although Poole *et al.* (2002) found an increase in $\delta^{13}C$ of up to 1.5‰ for peas charred between 130 and 700°C for 1 or 2 hours. For $\delta^{15}N$, a small increase due to charring is typical, but specific values are not always reported (Aguilera *et al.* 2008; Fiorentino *et al.* 2012; Kanstrup *et al.* 2012).

Aguilera *et al.* (2008) compared uncharred grains to grains charred at 250°C under both aerobic and anaerobic conditions. These were gradually heated for 2 hours, and then held at 250°C for 45 minutes (Ferrio *et al.* 2004). Their study found a strong (R^2=0.90) 1:1 correlation (slope=0.98) between the $\delta^{15}N$ ratio of charred and uncharred grains, regardless of treatment, with an offset of 0.68‰ between charred and uncharred grains. Fraser *et al.* (2013b) compared grains charred at a cooler temperature (230°C) but longer duration (24 hours) and recommended applying a conservative offset of 1‰. The average difference between their 39 charred–uncharred pairs from nine different species of cereals and pulses is 0.91‰ (paired *t*-test *t*=13.5, *P*<0.001, 95% confidence interval (CI) [0.77, 1.05‰]). Taken together, previous experiments, therefore, suggest that the offset in $\delta^{13}C$ and $\delta^{15}N$ between charred and uncharred samples is likely small, but not necessarily zero, and may depend on the duration and temperature of charring.

Intergrain isotopic variability

The goal of precisely measuring $\delta^{13}C$ and $\delta^{15}N$ of archaeological seeds is to extrapolate to the soil $\delta^{13}C$ and $\delta^{15}N$ and, thus, make inferences about the field conditions in which that crop was grown. This is problematic, however, given the inherent variability of seed isotope ratios. When C and N isotope ratios of grains from individual spikelets along wheat ears were measured, variations of up to 2.0% in $\delta^{15}N$ within a single ear were found (Bogaard *et al.* 2007), with up to 0.7‰ in $\delta^{13}C$ (Heaton *et al.* 2009). Peas within a single pod varied up to 0.7‰ in $\delta^{15}N$ (Fraser *et al.* 2013b). Even when measuring homogenized powder from large numbers of grains (~50) from three or four plots under identical conditions at the same site, the maximum range of $\delta^{15}N$ averaged 1.8±1.2‰ within a single growing condition.

While the problem of variability can be overcome by measuring and homogenizing even larger numbers of seeds, this is not always practical archaeologically when the material is limited. Kanstrup *et al.* (2012) concluded that measuring $\delta^{15}N$ from a homogenized powder of 10–15 grains adequately characterized the mean. For the six different fields in their study, the $\delta^{15}N$ standard deviation of six different homogenized powders of 10–15 grains ranged from 0.12 to 0.91‰. Manured fields tended to have higher variability. This study will test whether homogenizing 10 cereal grains (or five peas) reaches an adequate compromise between the need to obtain an average measurement while destroying a minimum amount of material, and we therefore sought to measure the variability of different groups of 10 grains (or five peas) as part of this experiment. Since the chemistry of charring takes place within the matrix of the seeds, it was not possible to avoid the problem variability by prehomogenizing seeds into flour prior to charring.

Methodological considerations

This study had five objectives: (1) to establish the limits in the range of temperatures and times associated with 'optimally charred' material, (2) to determine what offset in $\delta^{13}C$ and $\delta^{15}N$ ratios should be applied to correct charring archaeobotanical material, (3) to determine the uncertainty of this 'charring offset', (4) to measure interseed variability within a single growing context on a large scale and to examine how this variability might affect archaeological interpretations, and (5) to examine what effect the 'charring offset' may have when incorporating charred botanical remains in paleodietary reconstructions.

Variations in biochemical components of seeds make it entirely possible that different seeds could react differently to identical charring conditions (Braadbaart *et al.* 2005), but rather than attempting to closely match biochemical compositions and calculating charring offsets to extrapolate even to extinct landraces, we instead sought to capture the possible range of variations that might exist by combining the calculated effect of charring on a range of cereals and pulse crops: bread wheat, emmer wheat, einkorn, hulled barley, peas, and lentils. These crops were selected as being most relevant to research into food production in western Eurasia, and we would recommend that methods similar to ours be applied to crops with significantly different biochemical compositions, such as rice, condiments, or other food remains.

Methods

Material

For each taxon, plants were chosen from a single 'growing condition', meaning a single field or plot (where available), from the same farm, grown in the same year (Table 1). All crops were grown in unmanured soils, without chemical fertilizers, in temperate Europe. Although manuring and chemical fertilizers cause discernable alterations in $\delta^{13}C$ and $\delta^{15}N$ of plant, there is no evidence that plants grown under different conditions react differently to charring (Kanstrup *et al.* 2012). Different grains from harvest batch were previously analysed to determine the effect of manure on crop isotope ratios (Fraser *et al.* 2011).

Sampling

For each of the six main taxa and growing contexts listed above, a random sample of ~650 seeds was selected, which was subdivided into 13 subsamples of ~50. For each taxon, the groups of 50 seeds were charred under 12 different conditions, at 215, 230, 245, or 260°C for 4, 8, or 24 hours, with the thirteenth sample retained as the uncharred fraction. Each fraction was weighed before and after charring. From each charred fraction, three samples of 10 random grains were selected and each group of 10 grains homogenized separately for analysis. The A, B, and C replicates, thus, represent the effect of charring on three different groups, each consisting of 10 grains, from the same growing context (see Fig. 1). The remainder of the charred fractions was retained for morphological inspection. For peas, each replicate was made up of a homogenized powder of five seeds, and only A and B replicates were charred at 260°C because of the limited number of pea seeds available from what was known to be a single growing context.

A second experiment tested whether the presence or absence of the hull of hulled barley would have an effect on its overall isotopic composition and its response to charring. Since hulled barley can be found archaeologically with or without the palea/lemma (hulls) adhering to the grain, it was important to determine the degree of any potential isotopic difference. Approximately 150 hulled barley grains from the same plot grown in unmanured conditions at Askov, Denmark (see Table 1) were selected. Half were pearled by hand using a dull knife, scraping away the outer hull and bran until an effect similar to commercial pearled barley was achieved. The pearled and hulled grains were divided into two groups, half charred at 230°C for 24 hours and the other half uncharred. From each of the 4 groups, 3 groups of 10 grains were selected for homogenization into A, B, and C replicates, similar to the main charring experiment.

Charring

The grains and seeds for charring were loosely wrapped in aluminium foil packets, and buried in individual beakers of sand, after the method described by Fraser et al. (2013b). The availability of oxygen during charring has already been shown to have no effect on grain $\delta^{13}C$ and $\delta^{15}N$ (Aguilera *et al.* 2008; Fraser *et al.* 2013b; Kanstrup *et al.* 2012).

Samples were charred in a Gallenkamp Plus II electric oven. The oven was preheated, and the temperature was monitored throughout using a thermocouple buried in four dummy sand beakers placed at extreme corners of the oven. When the oven reached the desired temperature, the entire batch of samples was placed randomly in the oven, with samples being removed after 4, 8, and 24 hours. The temperature measured by the four thermocouples differed by no more than 4°C at any point, and we, therefore, assume that all grains in the oven were subjected to an adequately identical charring treatment.

Sectioning and microscopy

The grains and seeds were allowed to cool in their sand beakers until they reached room temperature. The subsample of each batch not subject to isotopic analysis was inspected under stereomicroscopy at 4×–16× magnification. A scalpel was used to cut one seed in half, perpendicular to the long axis of grains and perpendicular to the facet between the two cotyledons of pulses. This cross-section was also examined using a stereo microscope.

Table 1 Summary of growing conditions for cereal grains and legumes used for experimental charring

Taxon	Experiment
Bread wheat (*Triticum aestivum* L.)	Bad Lauchstädt long-term static fertilization experiment, Leipzig-Halle, Germany. Harvested 2001. Plot 18
Hulled barley (*Hordeum vulgare* var. *distichum* L.)	A single field from an organic farm in the Sault region of Provence, France. Harvested summer 2013
Pea (*Pisum sativum* L.)	Bad Lauchstädt long-term static fertilization experiment. Harvested 2008. Plot 18
Einkorn (*Triticum monococcum* L.)	Sutton Bonington short-term experiment, Nottinghamshire, UK. Harvested 2008. Field 18
Emmer (*Triticum dicoccum* L.)	Sutton Bonington short-term experiment, Nottinghamshire, UK. Harvested 2008. Field 3
Lentils (*Lens culinaris* L.)	Aroma Plantes, commercial organic farm, Sault, France. Purchased 2013
Barley comparison	
Hulled barley (*Hordeum vulgare* L. var. *distichum*)	Askov field experiment, Denmark. Plot 474. Harvested 2007
Naked barley (*Hordeum vulgare* L. var. *nudum*)	Askov field experiment, Denmark. Plot 513. Harvested 2008

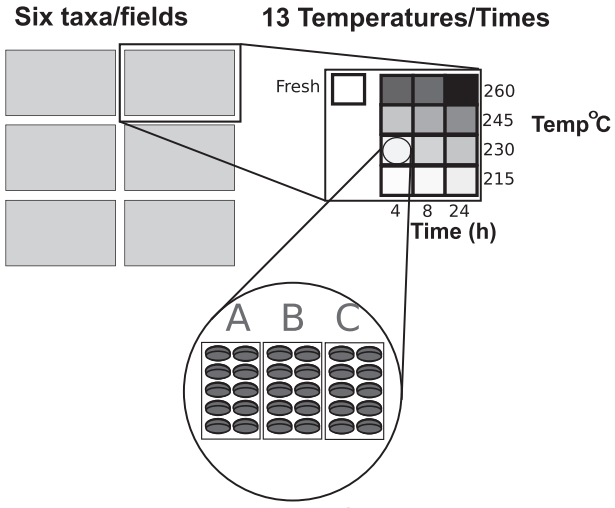

Figure 1 Sampling procedure for the charring experiment

Isotopic analysis

Once cool, three subsamples consisting of 10 seeds each (five for peas) were divided. Most samples were crushed into a homogeneous powder by hand using an agate mortar and pestle, but this was not possible for the peas, lentils, or uncharred grains, which were crushed in a Spex 2760 Freezer/Mill. The homogenized powders were weighed into tin capsules for analysis on a Sercon EA-GSL mass spectrometer. $\delta^{13}C$ and $\delta^{15}N$ were measured separately, with an internal alanine standard used to obtain raw delta values and for drift correction. Results were normalized to the Vienna Pee Dee Belemnite (VPDB) and ambient air (AIR) reference scales. Two-point normalization on the VPDB and AIR scales was calculated by interpolating the delta values of the samples against four replicates, each of two certified reference materials in each run. For $\delta^{13}C$, these were IAEA-C6 and IAEA-C7, and for $\delta^{15}N$, USGS40 and IAEA-N2. Supplementary Material 1 provides the secondary analytical data for each of the 21 runs that contributed data to this study. Measurement uncertainties for each sample were calculated based on the within-run variability of the reference materials and alanine standard and the known uncertainty of the two reference materials, as recommended by Ellison and Williams (2012), using the approximation method described by Kragten (1994). All calculations were performed using the statistical programming language R (3.0.1). The average measurement uncertainty for $\delta^{13}C$ was 0.062‰, with a range between 0.047 and 0.087‰. The average measurement uncertainty for $\delta^{15}N$ was 0.28‰, with a range between 0.17 and 0.41‰. The full analytical results are available as Supplementary Material 2.

Results

Physical changes at different temperatures and times

The majority of the charred material shows physical characteristics associated with optimally charred material, with nearly or entirely complete colour change and minimal physical distortion (distortion scale 1 or 2, Hubbard and al Azm 1990). The least charred batch 215°C for 4 hours with its nearly complete colour change is likely the lower limit for what might survive archaeologically, while the most charred samples 260°C for 24 hours are beginning to exhibit the more extreme distortions (distortion scale 3; Hubbard and al Azm 1990) that make identification to species difficult or impossible. These limits were relatively similar across all six taxa, although the specific nature of the changes was different for cereals compared to pulses. A reference guide to identifying optimally charred material is summarized in Table 2. Colour photographs of the different charred batches are available in Supplementary Materials 3–9.

Secondary data (mass loss, %N, %C, C/N ratio)

Charring resulted in significant mass loss, with all taxa losing approximately 25% of their original mass after charring for 4 hours at 215°C (see Fig. 2 and Table 3). Increasing the temperature and duration of charring increased the amount of mass lost, up to about 55% for seeds charred at 260°C for 24 hours. The majority of the charring temperatures and times resulted in 45–55% of mass loss. This is consistent with the pattern of mass loss achieved in previous charring experiments, but for shorter durations (60–180 minutes) at slightly higher temperatures (250–290°C) (Braadbaart *et al.* 2004b, 2004c). Previous research has suggested that this mass

Table 2 Summary of changes associated with the lower, average, and upper limits of the optimal charring window, representing seeds charred between 215 and 260°C for 4–24 hours

Taxon	Cereals (bread wheat, einkorn, emmer, barley)	Pulses (lentils, peas)
Lower limit	Minimal distortion; colour change still dark brown rather than black; hairs present at the apex of grain; and in cross-section, endosperm retains starch	Dark brown or nearly black colour, rather than black; testa firmly attached
Average	A slight swelling and rounding; complete black colour; and in cross-section, endosperm has a honeycomb of dense, empty 'cells'. For barley, the starchy endosperm material may leak out of the grain, forming a glossy pumice-like deposit that may not preserve archaeologically	Completely black colour change; testa begins to crack and may or may not be present. Cross-section texture is dense
Upper limit	The overall shape of the grain becomes very rounded, making identification difficult. Significant blisters appear in the epidermis and may even start to create large voids underneath. In cross-section, large fissures start to appear and texture of endosperm becomes more coarse. For barley, the amount of inner material that leaks out at higher temperatures reduced.	Cross-section texture becomes more coarse; testa very easily flaked off and is almost certainly absent in archaeological samples

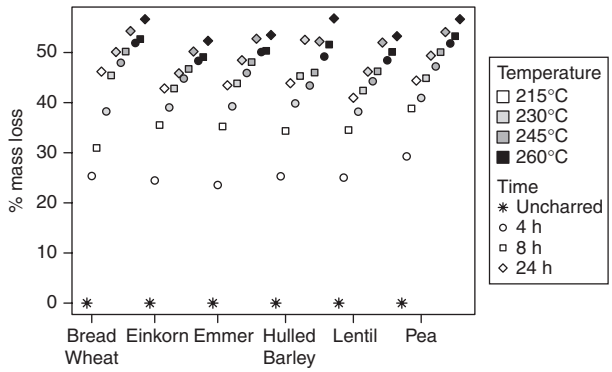

Figure 2 Summary of % mass loss for six different taxa charred at 12 different temperatures and times, from 4–24 hours at 215–260°C

is largely water (free and chemically bound) along with other volatile compounds, related to the breakdown of starch and formation of Maillard reaction products (Braadbaart *et al.* 2004a, 2004c; Styring *et al.* 2013). This is consistent with the pattern of increasing %C and %N due to charring (see Figs.3 and 4). The pattern of increasing %C and %N with increasing charring time and temperature is consistent across all six taxa, noting that peas and lentils have inherently higher N content compared to the cereals. Although the concentrations of C and N increase with charring, the molar ratio of C/N does not remain consistent (see Fig.5). For all taxa, C/N ratios decrease with increasing charring temperature and time, meaning that the increase in %N due to charring is proportionally higher than the increase in %C. This is likely due to larger amounts of C being lost during the charring process compared to N, and is consistent with the findings of previous studies (Fraser *et al.* 2013b; Styring *et al.* 2013).

Carbon isotope results

The uncharred taxa have $\delta^{13}C$ about −27 to −26‰, consistent with C3 vegetation. Comparing the charred $\delta^{13}C$ to the average of the uncharred replicates for each taxon (see Fig. 6), the offset due to charring is minimal compared to the variability. Carbon isotope ratios in bread wheat show increasing $\delta^{13}C$ offset with increasing charring temperature and time, a difference up to 0.25‰. This pattern is not repeated in any of the other taxa. The charred einkorn samples have slightly lower $\delta^{13}C$ than their uncharred counterparts (~0.1‰). Emmer charred at 230–245°C has an offset in $\delta^{13}C$ of up to 0.5‰, while those samples charred at 260°C have $\delta^{13}C$ ratios closer to the uncharred values. The variability in $\delta^{13}C$ within each individual batch (i.e. the standard deviation of the A, B, and C replicates for each temperature and time) is relatively consistent between taxa (see Table4). The average within-batch variability ranges from 0.14‰ for emmer to 0.27‰ for lentils, with an overall average of 0.19‰. Considering the charred data only, multiple linear regression with coefficients for temperature, time, and taxon shows no significant difference due to increasing temperature (Beta=−0.0008‰, standard error (SE)=0.0009‰, t=−0.875, P=0.38) and only a small effect due to increasing time (Beta=−0.004‰, SE=0.0018‰, t=2.35, P=0.02), equivalent to an increase of 0.016‰ every 4hours. The differences between the different charred batches are, therefore, relatively small. The differences between the charred and uncharred samples are discussed below.

Nitrogen isotope results

The uncharred seeds have $\delta^{15}N$ ratios that vary between 0 and 2‰, consistent with N-fixing pulses and unmanured cereals (Bogaard *et al.* 2013; Fraser *et al.* 2011; Szpak *et al.* 2014). Within-batch variability is larger for N than for C (see Table4), which may reflect both the larger instrumental uncertainty associated with $\delta^{15}N$ measurements as well as a wider range of $\delta^{15}N$ values in the isotopic ecosystem. Lentils show the smallest amount of variation, with an average within-batch standard deviation (A, B, and C replicates) of 0.23‰ compared to peas with 0.59‰. The average within-batch standard deviation for all replicates was 0.42‰. For emmer and lentil, there is a trend of increasing $\delta^{15}N$ offset with increasing charring temperature and time (see Fig. 7).

When the charred data are considered on their own, a multiple linear regression, including independent coefficients for temperature, time, and taxon, predicts a 0.12‰ increase in $\delta^{15}N$ for every 15°C (Beta=−0.008‰, SE=0.002‰, t=3.71, P=0.0003) and a 0.19‰ increase between 4 and 24 hours of charring (Beta=−0.01‰,

Table 3 Summary of % mass loss due to charring for different taxa

Temperature (℃)	Time (hours)	Bread wheat	Einkorn	Emmer	Hulled barley	Lentil	Pea
215	4	25.4	24.5	23.6	25.3	25.0	29.3
	8	31.0	35.5	35.2	34.4	34.5	38.8
	24	46.2	42.8	43.5	43.9	41.0	44.4
230	4	38.2	39.0	39.3	39.8	38.2	40.9
	8	45.4	42.8	43.8	45.3	42.4	44.9
	24	50.1	45.9	48.5	52.5	46.2	49.4
245	4	48.0	44.8	45.9	43.4	44.3	47.2
	8	50.2	46.7	48.1	46.0	46.2	50.1
	24	54.3	50.2	52.7	52.2	52.0	54.1
260	4	51.9	48.3	50.1	49.2	48.4	51.8
	8	52.7	49.1	50.3	51.6	50.1	53.3
	24	56.6	52.3	53.5	56.8	53.3	56.6

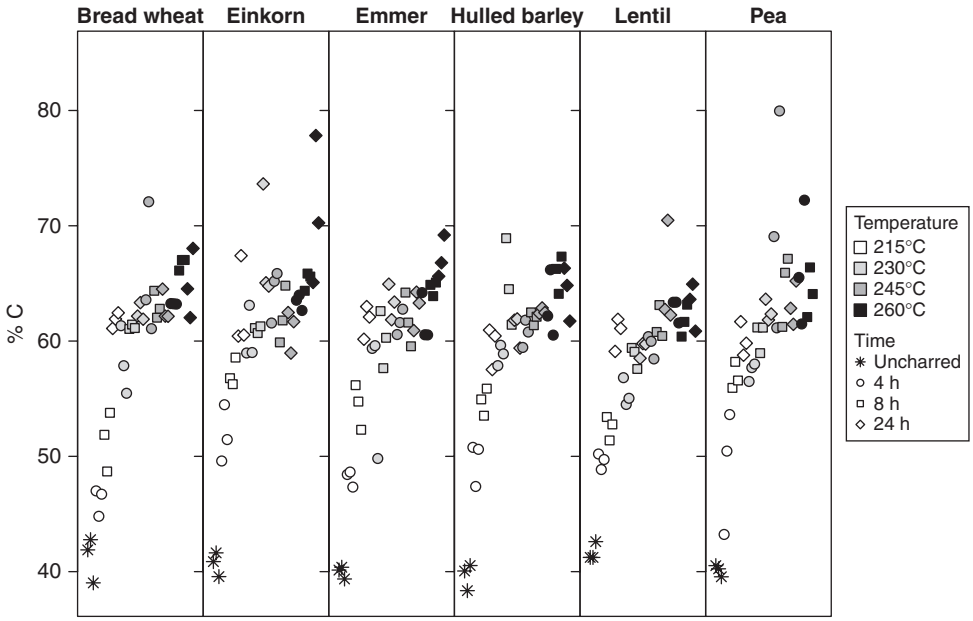

Figure 3 Changes in %C due to charring for six different taxa charred from 4–24 hours at 215–260°C

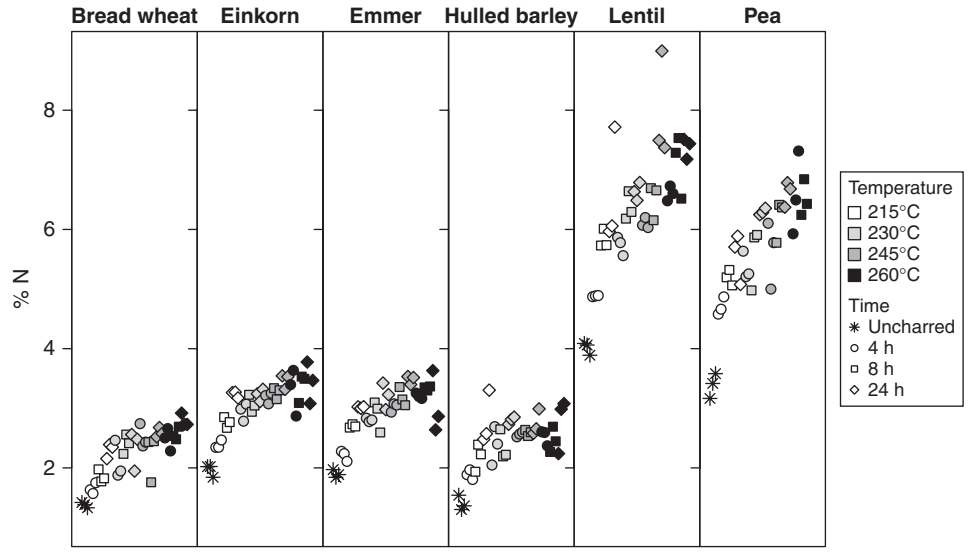

Figure 4 Changes in %N due to charring for six different taxa charred from 4–24 hours at 215–260°C

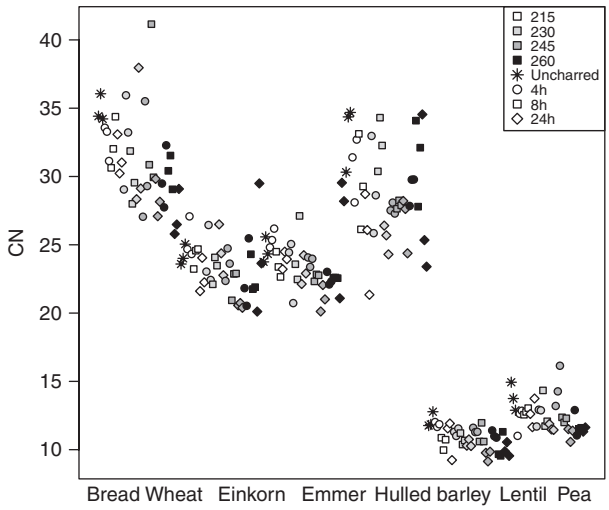

Figure 5 Changes in molar C/N ratio due to charring for six different taxa charred from 4–24 hours at 215–260°C

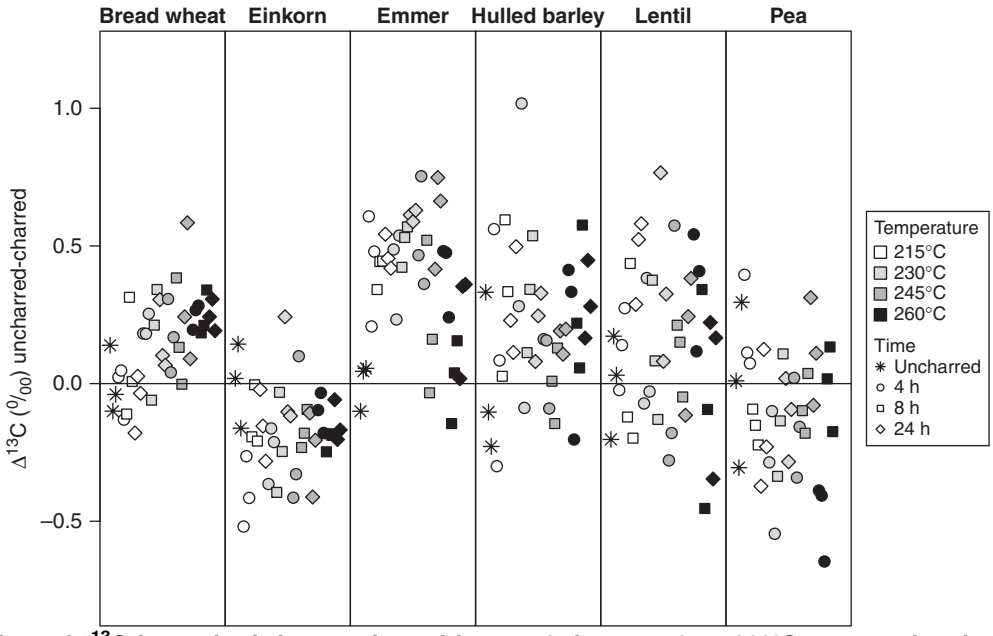

Figure 6 Charred $\delta^{13}C$ for each of six taxa charred from 4–24 hours at 215–260°C compared to the mean $\delta^{13}C$ of the three uncharred replicates

SE=0.004‰, *t*=2.36, *P*=0.019). There is, in other words, a demonstrable difference in the effect of charring on $\delta^{15}N$, depending on the precise temperature and duration, with the most charred samples (260°C for 24 hours) predicted to have 0.67‰ higher $\delta^{15}N$ than the least charred samples (215°C for 4 hours). The average $\delta^{15}N$ offset between charred and uncharred samples is discussed below.

Hulled vs. pearled barley

The uncharred pearled barley replicates were an average of 0.5‰ higher in $\delta^{15}N$ compared to the uncharred barley from the same plot that had retained its hull, a difference that is large compared to the variability of the three replicate measurements (see Table 5 and Fig. 8). The %C, %N, and C/N ratios of the hulled and pearled barley are similar despite the removal of the hull. If the higher $\delta^{15}N$ in the pearled barley is due to the removal of a lower $\delta^{15}N$ fraction, this fraction appears to have a %C and %N composition similar to the rest of the grain. On the other hand, the charred pearled and hulled barley had similar isotope ratios. The charred hulled barley had a $\delta^{15}N$ ratio 0.38‰ higher than the uncharred hulled barley, while the charred pearled barley was only 0.05‰ higher than its uncharred counterpart. These small increases in $\delta^{15}N$ due to charring are consistent with what was observed in the other six taxa. For $\delta^{13}C$, all differences between charred, uncharred, pearled, and hulled are negligible. Charring hulled barley has the same net isotopic effect as removing the hull of fresh barley.

Discussion

Calculating a charring offset

In calculating a general charring offset to encompass all relevant charring times and temperatures, we compared the $\delta^{13}C$ and $\delta^{15}N$ ratios of all charred seeds to the uncharred samples for all six taxa. This simplification is practically advantageous, given the difficulty of linking, by visual inspection, any single archaeological seed to a specific temperature or time. The analytical results (mass loss, $\delta^{13}C$, $\delta^{15}N$, %C, %N, C/N) suggest that while there may be variations within this window of charring temperatures (215–260°C) and times (4-24 hours), the six taxa behave similarly under similar charring conditions. Two multiple linear regression models were calculated for $\delta^{13}C$ and $\delta^{15}N$, comparing the effect of charring (all charred samples vs. all uncharred samples) with different coefficients for each taxon. For $\delta^{13}C$, the model, including coefficients for each of the six taxa, produced a good fit, with an adjusted R^2 of 0.91. The effect of charring (all charred vs. all not charred) was moderately significant (Beta=0.106‰, SE=0.056‰, *t*=1.913, *P*=0.057). The 95% CI for the estimated effect of charring was, therefore, between 0.003 and 0.22‰, with an average of 0.11‰. It should be noted that the average estimate effect of charring on $\delta^{13}C$, though small compared to typically reported analytical precision, is statistically significant despite the variability of individual sample measurements in this experiment.

The larger amount of variation in $\delta^{15}N$ (whether due to ecological or analytical reasons) resulted in a poorer model fit, with an adjusted R^2 of 0.41, but the inclusion of a coefficient for charring (all charred vs. all not charred) improves upon the fit of a model with coefficients for the individual taxa alone (adjusted R^2=0.39). The inclusion of the charring coefficient is significant (Beta=0.31‰, SE=0.13‰, *t*=2.37, *P*=0.019) with a 95% CI between 0.05 and 0.57‰. This uncertainty largely represents the potential range of offsets produced by different charring temperatures and times, since, as

Table 4 Average $\delta^{13}C$, $\delta^{15}N$, %C, %N, and CN for the A, B, and C replicates of the 13 batches of charring temperatures and times for six different taxa

Taxon	Temp (℃)	Time (hours)	$\delta^{13}C$ (‰)	1σ	%C	$\delta^{15}N$ (‰)	1σ	%N	C/N
BW	0	0	−27.15	0.12	41.23	2.09	0.11	1.38	29.91
	215	4	−27.17	0.10	46.17	1.86	0.49	1.65	28.00
		8	−27.08	0.22	51.45	2.07	0.48	1.86	27.72
		24	−27.21	0.11	61.82	2.32	0.49	2.30	26.95
	230	4	−26.94	0.04	58.22	1.65	0.11	2.10	28.06
		8	−26.99	0.21	61.20	2.43	0.73	2.40	25.55
		24	−26.99	0.13	62.46	2.28	0.31	2.33	27.26
	245	4	−26.98	0.13	65.58	1.86	0.36	2.51	26.25
		8	−26.98	0.20	63.06	1.61	0.65	2.21	29.13
		24	−26.84	0.25	62.94	2.16	0.53	2.59	24.30
	260	4	−26.90	0.05	63.23	2.29	0.10	2.48	25.57
		8	−26.91	0.08	66.73	2.10	0.09	2.57	26.00
		24	−26.90	0.06	64.87	2.45	0.23	2.79	23.25
EI	0	0	−27.05	0.15	40.69	1.19	0.24	1.96	20.76
	215	4	−27.45	0.13	51.84	1.14	0.51	2.39	21.74
		8	−27.18	0.11	57.20	1.51	0.06	2.76	20.71
		24	−27.20	0.13	62.79	1.36	0.10	3.24	19.40
	230	4	−27.29	0.11	60.36	1.76	0.30	2.95	20.55
		8	−27.27	0.18	61.03	1.68	0.49	3.07	19.90
		24	−27.04	0.20	67.82	1.79	0.55	3.22	21.04
	245	4	−27.26	0.28	64.20	1.56	0.64	3.18	20.20
		8	−27.22	0.07	62.16	1.75	0.59	3.26	19.06
		24	−27.29	0.16	61.04	1.42	0.69	3.46	17.63
	260	4	−27.15	0.07	63.39	1.29	0.40	3.30	19.38
		8	−27.25	0.04	65.27	1.09	0.12	3.37	19.42
		24	−27.19	0.08	71.05	1.12	0.84	3.44	20.93
EM	0	0	−25.84	0.09	39.96	0.87	0.11	1.90	21.05
	215	4	−25.41	0.20	48.13	1.23	0.39	2.21	21.81
		8	−25.43	0.06	54.41	1.42	0.41	2.70	20.15
		24	−25.36	0.06	61.75	1.43	0.47	3.02	20.47
	230	4	−25.42	0.16	56.26	1.09	0.04	2.80	20.06
		8	−25.33	0.08	60.18	1.68	0.18	2.90	20.90
		24	−25.23	0.02	63.39	1.52	0.79	3.21	19.79
	245	4	−25.31	0.20	61.64	1.28	0.34	3.02	20.41
		8	−25.62	0.28	61.79	1.78	0.63	3.19	19.41
		24	−25.23	0.17	62.82	2.23	0.59	3.48	18.05
	260	4	−25.44	0.14	61.76	2.22	0.67	3.21	19.27
		8	−25.82	0.15	64.62	1.76	0.28	3.34	19.36
		24	−25.59	0.20	67.20	1.42	1.07	3.04	22.52
HBH	0	0	−26.61	0.29	39.65	0.51	0.29	1.40	28.39
	215	4	−26.50	0.43	49.59	0.74	0.31	1.89	26.34
		8	−26.30	0.28	54.78	0.38	0.30	2.18	25.29
		24	−26.33	0.20	59.64	0.23	0.26	2.78	21.75
	230	4	−26.21	0.56	58.80	1.08	0.50	2.38	24.98
		8	−26.28	0.21	64.95	0.93	0.34	2.35	27.70
		24	−26.40	0.13	61.04	1.26	0.57	2.80	21.83
	245	4	−26.54	0.14	60.68	0.99	0.53	2.56	23.68
		8	−26.62	0.14	61.99	0.72	0.22	2.59	23.94
		24	−26.45	0.05	62.58	0.76	0.38	2.74	22.92
	260	4	−26.43	0.33	62.96	0.89	0.55	2.52	24.97
		8	−26.33	0.27	65.89	0.49	0.50	2.47	26.85
		24	−26.32	0.14	64.29	0.64	0.80	2.77	23.80
LE	0	0	−26.30	0.19	41.69	0.08	0.22	4.01	10.40
	215	4	−26.17	0.15	49.60	0.35	0.21	4.88	10.16
		8	−26.26	0.35	52.52	0.69	0.08	5.83	9.02
		24	−25.84	0.16	60.69	0.70	0.23	6.58	9.35
	230	4	−26.21	0.25	55.45	0.83	0.30	5.73	9.67
		8	−26.19	0.25	58.68	0.60	0.13	6.37	9.22
		24	−25.91	0.35	59.34	1.15	0.34	6.64	8.95
	245	4	−26.26	0.47	59.60	0.98	0.25	6.10	9.77
		8	−26.20	0.14	61.45	1.01	0.34	6.50	9.47
		24	−26.13	0.26	65.16	1.45	0.29	7.95	8.22
	260	4	−25.94	0.22	62.77	1.31	0.25	6.60	9.51
		8	−26.37	0.40	61.74	1.60	0.01	7.11	8.72
		24	−26.29	0.31	63.14	1.22	0.29	7.38	8.57
PE	0	0	−27.59	0.30	40.11	1.76	0.48	3.39	11.88
	215	4	−27.39	0.18	49.10	1.21	1.61	4.70	10.43
		8	−27.74	0.07	56.91	1.06	0.53	5.19	10.96
		24	−27.75	0.26	60.09	1.14	0.28	5.56	10.86
	230	4	−27.90	0.22	57.40	0.97	0.39	5.37	10.71

Table 4. *Continued*

Taxon	Temp (℃)	Time (hours)	$\delta^{13}C$ (●)	1σ	%C	$\delta^{15}N$ (●)	1σ	%N	C/N
		8	−27.71	0.22	60.43	1.70	0.92	5.58	10.90
		24	−27.71	0.15	62.60	1.63	0.64	6.30	9.94
	245	4	−27.75	0.18	70.06	0.91	0.43	5.63	12.46
		8	−27.67	0.11	64.76	1.20	0.34	6.19	10.47
		24	−27.47	0.20	63.17	2.12	0.51	6.61	9.56
	260	4	−27.98	0.01	63.50	1.72	0.51	6.21	10.26
		8	−27.90	0.47	67.15	2.22	0.67	6.78	9.91
		24	−27.61	0.22	65.23	2.81	0.43	6.64	9.83

described above, there are no substantive differences in $\delta^{15}N$ between the 215°C at 4 hours group and the uncharred group (see Fig. 7).

The charring offset for $\delta^{15}N$, we calculate here, is significantly lower than the 1‰ 'worst-case' estimate reported by Fraser *et al.* (2013b), based on experiments done at 230°C for 24 hours. Aguilera *et al.* (2008) and Kanstrup *et al.* (2012) did not recommend applying a charring offset, on the basis of experiments done at slightly higher temperatures (250°C) for shorter durations (2–6 hours), although the dataset reported by Aguilera *et al.* (2008) has an average offset of 0.68‰ due to charring, while the data reported by Kanstrup *et al.* (2012) show small but significant effects due to temperature and time similar to those reported here.

Our experiment considered the possible variations that different charring temperatures and times could introduce, within a window that results in seeds with the same minimally distorted characteristics. The average offset observed is due to the averaging of larger offsets (1‰ or more) from more extreme temperatures and times with the negligible offsets of less extreme burns. Given the visual similarity of all 'optimally charred' materials regardless of temperature or time, we recommend the average offset for all materials within this range of temperatures for future use.

Using the charring offset

One of the most important applications of accurate archaeobotanical $\delta^{15}N$ (and $\delta^{13}C$) measurement is in estimating the $\delta^{15}N$ value of the plant-based component of human diet directly, rather than relying on indirect estimates from local herbivores. It has long been observed (Hedges and Reynard 2007) that estimates of the animal protein fraction of the diet that rely on comparisons between local herbivore and human $\delta^{15}N$ may be problematic. The observation that agricultural practices, such as manuring, can increase cereal $\delta^{15}N$ by as much as 2–5‰ (Bogaard *et al.* 2007; Fraser *et al.* 2011) and that charred crop remains preserve the original $\delta^{15}N$ ratio (Fraser *et al.* 2013b; Styring *et al.* 2013) has resulted in new opportunities to reconstruct crop management strategies and dietary practices. Comparisons of crop and animal $\delta^{15}N$ have demonstrated that the interrelationship of animal husbandry and agriculture dates back to the origins of

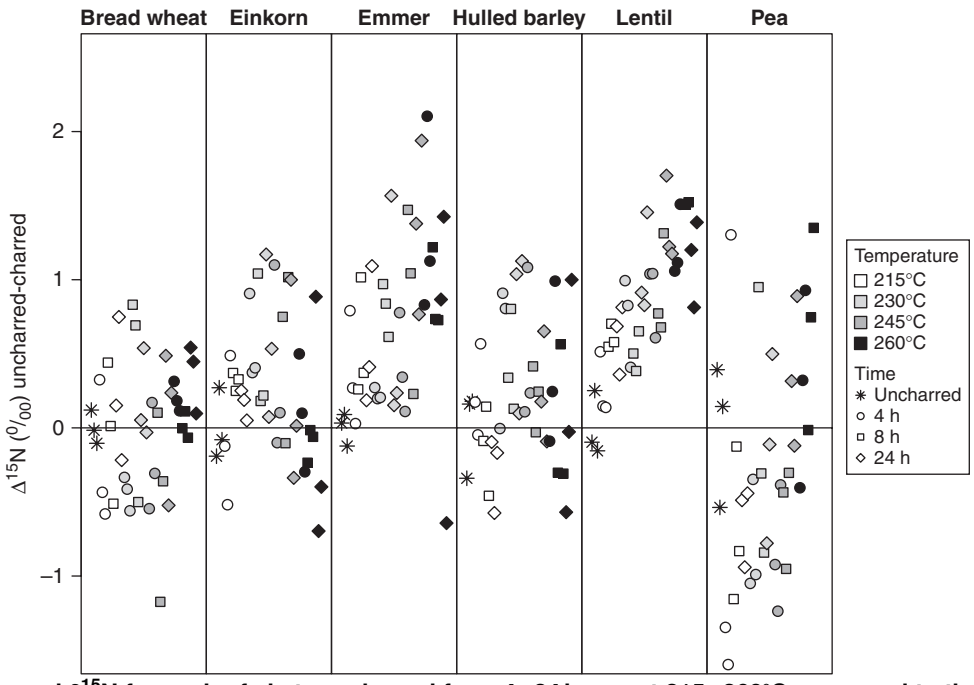

Figure 7 Charred $\delta^{15}N$ for each of six taxa charred from 4–24 hours at 215–260°C compared to the mean $\delta^{15}N$ of the three uncharred replicates

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Table 5 Residual standard error (SE) for taxon-specific multiple regression of the formula y = temp(x) + time(x), where y=$\delta^{13}C$ or $\delta^{15}N$

Taxon	$\delta^{13}C$ (●)	$\delta^{15}N$ (●)
Bread wheat	0.16	0.42
Einkorn	0.15	0.48
Emmer	0.22	0.56
Hulled barley	0.26	0.48
Lentil	0.28	0.36
Pea	0.23	0.75

agriculture in SW Asia (Bogaard *et al.* 2013). Where crops with relatively high $\delta^{15}N$ compared to animal collagen are found (e.g. Vaiglova *et al.* 2014), dietary reconstructions result in a higher estimate for the contribution of plants. At Vaihingen, Fraser *et al.* (2013a) showed that including the charring-corrected cereal $\delta^{15}N$ in a simple model of $\delta^{15}N$ resulted in a shift in the estimated animal protein fraction, estimated from the adult human mean $\delta^{15}N$, from 60 to 29%. Their model relied on a more conservative estimate of the charring offset of 1‰, meaning that the estimated uncharred plant $\delta^{15}N$ was made lower and closer to what would have been the estimated value based on the faunal collagen in accordance with the 'standard model'. Based on our more recent charring experiments, we are confident that the actual subtraction need only be about 0.31‰, with probable worst-case estimate of as much as 0.57‰. Using this new offset effectively increases the apparent uncharred plant $\delta^{15}N$, making the difference between the estimated cereal and fodder values even greater. This small adjustment has a significant effect on the estimated amount of animal protein, making its value likely less than 10% of the dietary protein fraction (see Fig. 9). Using the new offset for Vaihingen, it becomes realistically possible for a combination of cereals (with slightly higher $\delta^{15}N$ than previously thought) and low-$\delta^{15}N$ pulses to have been consumed, as part of a diet that also contained small amounts of animal protein (about 25‰, depending on the relative ratio of cereals to pulses). Further refinements to the paleodietary model, increasing the human-fauna $\delta^{15}N$ trophic offset to +5–6‰ also argues for a significant consumption of pulses at this site (Bogaard In press).

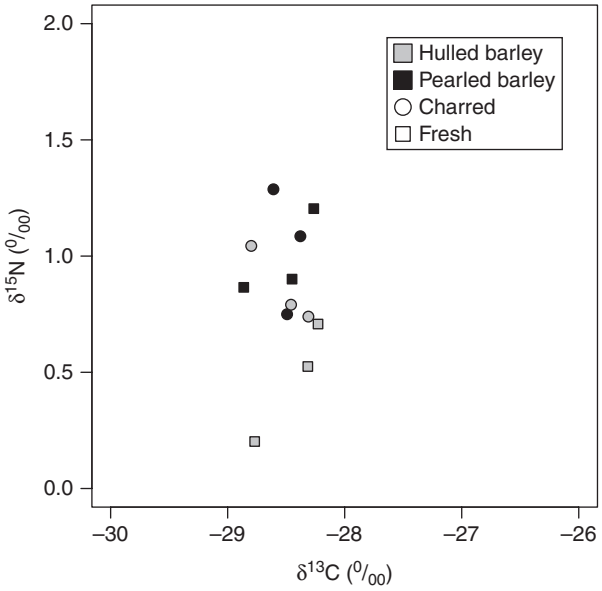

Figure 8 Comparison of $\delta^{13}C$ and $\delta^{15}N$ for hulled and pearled barley, both fresh and charred (at 230°C for 24 hours). Each data point represents a homogenized sample of 10 grains. All grains were grown in the same plot, under the same unmanured treatment, at Askov, Denmark.

Variability and issues of comparability

Our study homogenized 10 seeds per measurement (five for peas), a quantity which is minimally destructive to archaeology material, while ensuring sufficient N for $\delta^{15}N$ analysis and adequately representing the average isotopic ratio of the context. We estimated the average variability for $\delta^{13}C$ and $\delta^{15}N$ by examining the residual SE of a multiple regression model that accounted for the effect of temperature and time. The taxon-specific residual SEs were between 0.16 and 0.28‰ for $\delta^{13}C$ and between 0.36 and 0.75‰ for $\delta^{15}N$ (see Table6). A figure of 0.25‰ for $\delta^{13}C$ and 0.5‰ for $\delta^{15}N$ provides a rough guide to the expected population SE of a given growing context, and therefore, a 95% CI (1.96×SE) of ±0.5‰ for $\delta^{13}C$ and ± ~ 1‰ for $\delta^{15}N$ to account for variability within a single growing context. Since the SE is calculated by dividing by the square root of the sample size, this uncertainty would theoretically become larger when smaller numbers of seeds are crushed, and decreasing the sample size to five would increase the uncertainty with which the mean could be estimated by about 40% (see Fig.10).

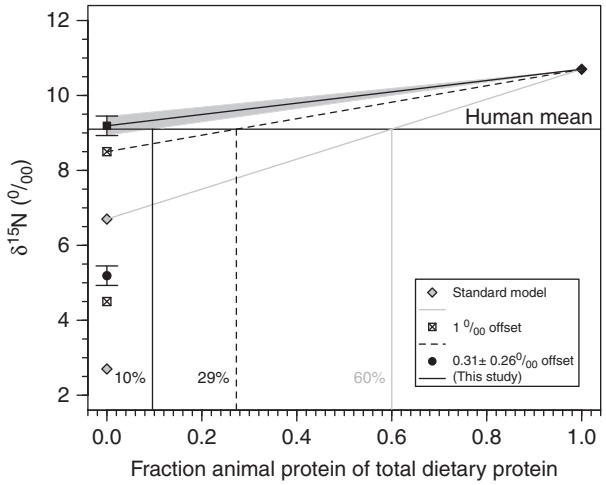

Figure 9 An example of a paleodietary reconstruction based on mean human collagen $\delta^{15}N$ at Neolithic Vaihingen, Germany, relative to local crop $\delta^{15}N$, using a 1‰ charring offset correction (from Fraser *et al.* 2013a) compared to the new smaller offset of 0.31 ± 26‰ (this study). Also shown is the calculation based on an 'inferred' crop $\delta^{15}N$ from herbivore collagen, also known as the Standard Model (after Hedges and Reynard 2007)

Table 6 Comparison of $d^{13}C$, $d^{15}N$, %C, %N, and C/N ratio for hulled vs. pearled barley from the same experimental plot

Taxon	Temp (℃)	Time (hours)	$\delta^{13}C$ (●)	1σ	%C	$\delta^{15}N$ (●)	1σ	%N	C/N
HBA	0	0	−28.44	0.29	40.56	0.48	0.26	1.36	30.01
HBA	230	24	−28.52	0.25	63.03	0.86	0.16	2.47	25.57
PBA	0	0	−28.53	0.31	40.60	0.99	0.19	1.31	31.09
PBA	230	24	−28.49	0.12	62.76	1.04	0.27	2.58	24.42

HBA: hulled barley; PBA: pearled barley.

However, this suggested relationship between crushed number of seeds and uncertainty should be used as a rough guideline only, as illustrated by the variability of peas ($N_{crushed}$=5) we calculated from this experiment, a figure which is about twice that of lentils ($N_{crushed}$=10) for $\delta^{15}N$ (as theoretically expected) but not for $\delta^{13}C$. The more important point is that interpretations of $\delta^{13}C$ and $\delta^{15}N$ measurements should consider that results are only 95% likely to encompass the true population mean if their confidence range is extended ±0.5‰ (for $\delta^{13}C$) or ±1.0‰ (for $\delta^{15}N$) and that analyses based on <5 seeds may be too variable to merit interpretation.

Conclusions

Small offsets in $\delta^{13}C$ and $\delta^{15}N$ of charred archaeobotanical remains need to be taken into account when comparing fresh and charred material: +0.11‰ with a 95% CI of 0.003–0.22‰ for $\delta^{13}C$ and +0.31‰ with a 95% CI of 0.05–0.57‰ for $\delta^{15}N$. This value is lower than the 1‰ difference reported by Fraser *et al.* (2013b) but is higher than the figure of 0‰ recommended by Kanstrup *et al.* (2012) and Aguilera *et al.* (2008). The precise offset is highly dependent on the temperature of charring (0.19‰/15°C among charred seeds), but the probable average offset calculated here reflects the range of temperatures (215–260°C) and times (4–24hours) that results in minimal distortion to seeds. Such optimally preserved material is ideal for isotopic analysis because of its high likelihood of preservation while also retaining the physical characteristics making it identifiable to species. The more extreme distortion characteristics of 'overcharred' material result in seeds that are less likely to preserve and more difficult to identify, meaning that analysis of such material is unlikely to prove informative, and should, moreover, be avoided because the isotopic offset due to charring is likely larger than that reported here.

An average charring offset was calculated by assuming that all 12 different charring batches were equally representative of potential archaeological material. We also assumed that calculating an average effect for similar taxa would be more valuable than attempting taxon-specific calculations, an assumption that was partly validated by the broad similarities in response to charring among all four cereals, both pulses, and all six taxa together. A final factor considered was the probability that differences between charred and uncharred seeds could be due to random variability, even for seeds grown in the same context.

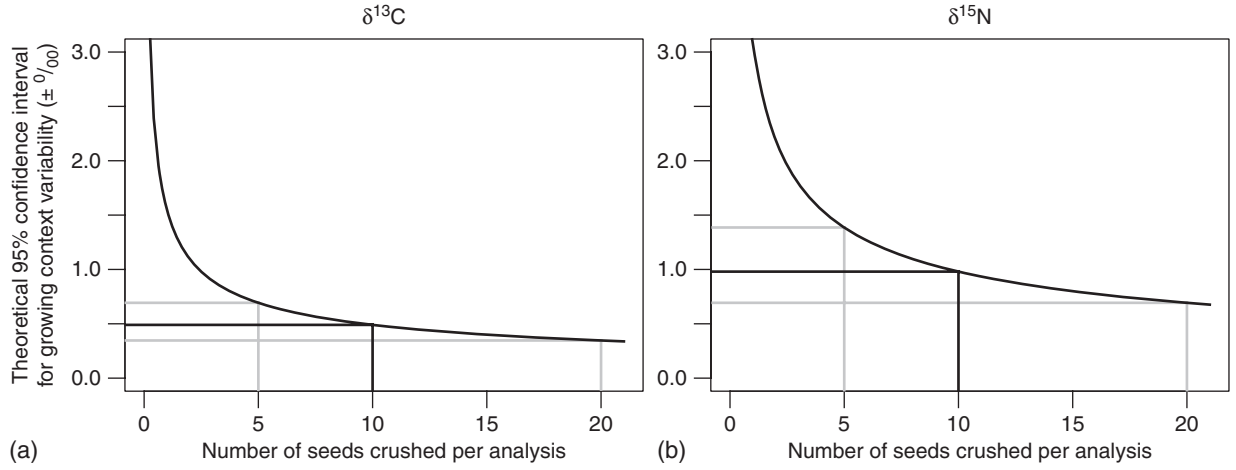

Figure 10 Illustration of the effect of sample size, specifically number of seeds crushed per analysis (*x*), on the size of the 95% CI (±‰) for estimating the population mean from which those seeds are drawn. It is based on an estimated population standard error (SE) of 0.25‰ for $\delta^{13}C$ and 0.5‰ for $\delta^{15}N$ from this study. This estimated value is equivalent to taking multiple random samples from the population of grains from a single growing context of size *n* = 10, calculating the mean of each group of 10, and then calculating the standard deviation of the distribution of means, and is thus equivalent to the population SE. The actual sample standard deviation (*s*) is equal to SE(*n*), and is, therefore, 0.25(10) in example A. Since the 95% CI is equal to ±1.96 × (Sn)), the two lines plotted are (A) $y = 1.96 \times (0.25(10)/x$ and (B) $y = 1.96 \times (0.5(10)/x$

This variability was relatively consistent, within a factor of 2, across the six taxa. We conclude that large numbers of seeds should be homogenized per analysis to obtain a reliable averaging of the inherent variability. Even using as many as ~10 seeds/analysis, the true average value of the growing condition will likely fall only within ±0.5‰ ($\delta^{13}C$) or ±1.0‰ ($\delta^{15}N$) of the measurement obtained. We suggest that $\delta^{13}C$ and $\delta^{15}N$ measurements of charred archaeobotanical material should be reported as uncorrected values relative to VPDB and AIR, and that when making comparisons between charred and fresh material, the mean charring offset plus the upper and lower 95% CI limits are all examined.

Conflicts of interest

The authors declare no conflicts of interest.

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Erika Nitsch is a postdoctoral research assistant at the University of Oxford, working as part of the "Agricultural Origins of Urban Civilization" project, funded by the European Research Council. Her research applies stable isotope analysis to preserved crop, animal and human remains in order to reconstruct the nature, scale and significance of farming in the urbanization of the Aegean during the Bronze Age.

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