Reconstructing Herbivore Diets: A Multivariate Statistical Approach To Interpreting Compound-Specific Isotope Values

Mickey Hong Yi Chen (✉ mickey.chen@mail.utoronto.ca)

University of Toronto - Mississauga <https://orcid.org/0000-0002-8787-2033>

Iain P. Kendall

University of Bristol

Richard P. Evershed

University of Bristol

Amy Bogaard

University of Oxford

Amy K. Styring

University of Oxford

Research Article

Keywords: CSIA-AA, Palaeodiet, Bayesian statistics, Amino acids, nitrogen isotopes

Posted Date: November 1st, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-836199/v1>

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Abstract

Stable nitrogen (N) isotope analysis of bulk tissues is a technique for reconstructing the diets of organisms. However, bulk nitrogen isotope ($\delta^{15}\text{N}$) values can be influenced by a variety of metabolic and environmental factors that can confound accurate dietary reconstruction. Compound-specific isotope analyses of amino acids (CSIA-AA) have demonstrated the power of the approach in understanding how the $\delta^{15}\text{N}$ values of bulk collagen are assembled from the constituent AAs. Furthermore, by connecting these AA $\delta^{15}\text{N}$ values within a robust biochemical framework interpretation of diet and environment are greatly enhanced. Several new proxies have emerged, built around selected AAs; however, the interconnectedness of AA biosynthetic pathways means that patterning of $\delta^{15}\text{N}$ values across a wider suite of collagen AAs will occur under different environmental or dietary influences. This work seeks to test this idea by situating CSIA-AA within a robust statistical framework using principal component analysis (PCA) and Bayesian statistics to increase the interpretability of a wider range of AA $\delta^{15}\text{N}$ values in terms of reconstructing herbivore diet. The model was tested using wild and domestic herbivores from the Neolithic settlements of Çatalhöyük (Turkey), Makriyalos (Greece), and Vaihingen (Germany) as case studies. It was found that at Makriyalos there was a sharp separation between domesticated and wild herbivores, which was present to a lesser extent at Çatalhöyük and not observed at Vaihingen. The case studies presented in this work demonstrate that multivariate statistical treatment of CSIA-AA data can deliver new insights into herbivore diet, exceeding those achievable with the Bayesian model.

Introduction

Dietary reconstruction through isotope analysis is a powerful technique which can disentangle diets of individuals living in complex ecosystems. Isotope analysis of bulk tissues (referred to here as the 'bulk' method) has been used for reconstruction of animals diets to explore herding and foddering practices, as well as the interplay between crop cultivation and animal husbandry (Hamilton et al. 2009; Makarewicz 2014). This most often involves determining the stable carbon (C) and nitrogen (N) isotope values of the extracted organic components of bone (collagen) or dental remains (dentine) and using those isotope values for distinguishing between various diet sources for reconstruction. C isotope ($\delta^{13}\text{C}$) values can be used to distinguish between C_3/C_4 plant diets (Farquhar et al. 1989) and N isotope ($\delta^{15}\text{N}$) values are commonly used as a trophic level indicator (Hedges and Reynard 2007). Based on controlled studies of rodents and pigs, a pure C_3 diet can result in $\delta^{13}\text{C}$ values of bone collagen between -22‰ and -14‰ , while a pure C_4 diet can lead to $\delta^{13}\text{C}$ values between -16‰ to -8‰ (Howland et al. 2003; Froehle et al. 2010; Webb et al. 2017). The ^{15}N -enrichment of a consumer's tissues over their diet is commonly cited as 4‰ (Hedges and Reynard 2007), but this can vary considerably from 2‰ – 6‰ (DeNiro and Epstein 1981; O'Connell et al. 2012). Moreover, a host of metabolic differences and variation in source dietary isotope values can affect bulk C and N isotope values in the consumer. C and N isotope values depend on the metabolism of the consumer species, which may be different in, for example, ruminants compared with other mammals (Kendall et al. 2017). Plant N isotope values can be affected by anthropogenic factors,

with use of manure or midden materials in crop cultivation potentially resulting in elevated $\delta^{15}\text{N}$ values (Bogaard et al. 2007; Styring et al. 2014b). Complicating the picture further, environmental variables also play a role. Plant tissues become ^{13}C -depleted due to CO_2 recycling under forest cover, lowering $\delta^{13}\text{C}$ values by up to 5‰ (Van der Merwe and Medina 1991; Bonafini et al. 2013). Other environmental factors can include aridity, which can elevate $\delta^{15}\text{N}$ values (Craine et al. 2009) and favour C_4 plants over C_3 species. Indirect effects, such as salinity, can favour C_4 plant growth by restricting freshwater availability (Chmura and Aharon 1995). C_3 and C_4 plants have been found to show some differences in N isotopic values, with a slight increase in bulk $\delta^{15}\text{N}$ isotope values of C_4 plants over those of C_3 plants by ca. 1.5‰ (Hare et al. 1991).

Attempts to resolve these limitations in bulk isotope analysis led to the development of compound-specific isotope analysis of amino acids (CSIA-AA). CSIA-AA involves breaking down peptide linkages between AAs, after which the isotopic compositions of individual AAs can be analyzed separately (Howland et al. 2003; Corr et al. 2007; Dunn et al. 2011; Styring et al. 2012; Kendall and Evershed 2020). By analyzing AAs individually, isotopic variability in the dietary source can be evaluated independently from consumer metabolism through distinguishing between AAs which become enriched in the heavy isotope with increasing trophic level (trophic group AAs) and those which do not (source group AAs) (Popp et al. 2007; O'Connell 2017; Ohkouchi et al. 2017). Current efforts in applying CSIA-AA to dietary reconstruction have tended to focus on resolving the diets of higher-order consumers, such as humans and aquatic organisms and estimating trophic positions (Chikaraishi et al. 2009; Naito et al. 2010; Steffan et al. 2013; Chikaraishi et al. 2014; Nielsen et al. 2015; Itahashi et al. 2019). Much less attention has been given to diets of past herbivores, which are important for understanding the interplay between crop and animal husbandry (through identifying foddering) and for gaining insights into herding strategies (Kendall et al. 2017). The majority of studies to date have focused on only three AAs by calculating the difference between Glx, the averaged amino N $\delta^{15}\text{N}$ signal of glutamic acid and glutamine, and phenylalanine (i.e. $\Delta^{15}\text{N}_{\text{Glx-Phe}}$) which may be under-exploiting the potential resolution that can be achieved using this approach (O'Connell 2017).

To explore the untapped potential that likely resides within the full suite of protein AAs, we investigate correlations between multiple AA $\delta^{15}\text{N}$ values in crops (cereal grains, rachis, legumes) and non-crops (herbaceous and woody plants). We use these correlations to differentiate between potential dietary sources and compare those dietary sources against trophic adjusted AA $\delta^{15}\text{N}$ values of archaeological herbivores through principal component analysis (PCA). We then apply a Bayesian model (FRUITS) that is increasingly used in archaeological studies to reconstruct diet (Fernandes et al. 2014). A predominance of any dietary plant type would imply particular management strategies and subsequent ecological implications. The use of crops as fodder would imply close integration of crop and animal husbandry, whereas diets of woody plants might suggest exploitation of the surrounding woodland. To assess the performance of these statistical tools, we test using AA N isotopic data from modern cattle (*Bos taurus*) fed on known diets (Kendall et al. 2017) in addition to wild and domestic herbivores from the Neolithic

sites of Çatalhöyük (Turkey), Makriyalos (Greece), and Vaihingen an der Enz (Germany) (Styring et al. 2015).

The three Neolithic sites have been well documented through previous isotopic analysis of bulk bone collagen, and there are clear expectations of herbivore diet which makes these sites ideal for testing new models. Çatalhöyük is located on the Konya plain in central Anatolia where, during the Neolithic, an extensive settlement developed with a domestic herbivore population dominated by sheep (*Ovis sp.*) and to a lesser extent, goats (*Capra sp.*) (Twiss et al. 2021; Wolfhagen et al. 2021). The surrounding plains consisting of C₃ and C₄ plants are supported by a dryland anabranching fluvial regime (Ayala et al. 2017). Elevated $\delta^{13}\text{C}$ values of aurochs (*Bos primigenius*), sheep, and goat bone collagen, coupled with C₄ plant species recovered from sheep dung, suggest C₄ plant contributions to diet (Pearson et al. 2021). Moreover, strontium isotope values from domesticated herbivores suggest herding was mainly confined locally to the plains (Bogaard et al. 2014). The second site, Makriyalos, is located in present day Greece two kilometers west of the Thermaic Gulf and 15 kilometers northeast of the Pieria mountains. The site is flanked by coastal saline marshes 7–8 kilometers to the north and south. The domestic cattle at this site have noticeably ^{13}C -enriched bone collagen ($\delta^{13}\text{C}$ mean: $-16.0 \pm 2.1\text{‰}$, $n = 19$) which Vaiglova et al. (2018) has suggested could be due to feeding in saline marshes. Strontium isotope evidence does not suggest use of migrative herding strategies (Vaiglova et al. 2018). In contrast to the previous two sites, Vaihingen an der Enz in south-western Germany was surrounded by a wooded environment, where domestic cattle dominated the livestock spectrum (Bogaard et al. 2017). Strontium isotope evidence indicates that cattle were likely herded in the local valley during the summer and kept closer to the settlement during winter (Bogaard et al. 2017). Overlapping C and N isotopic values between deer (*Cervus elaphus*) and domestic cattle indicate access to similar dietary resources over time (Fraser et al. 2013).

The aims of this study include:

1. Use of PCA to explore metabolic relationships between AAs and inform which ones will be most useful in resolving the contribution of dietary resources to herbivore diet,
2. Assessment of the predictive potential of a Bayesian model to reconstruct herbivore diet using a control group of modern cattle AA $\delta^{15}\text{N}$ values,
3. Investigation into what these approaches can reveal about wild and domestic herbivore diet at 3 archaeological sites.

Materials And Methods

This study utilizes AA $\delta^{15}\text{N}$ values determined by Styring et al. (2014a; b; 2015) and Kendall et al. (2017; 2019). Similar derivatization, instrumentation, and data processing protocols were used such that the datasets are compatible for combined analysis. The most essential sampling conditions are summarized here, with precise details available in the publications.

The dataset is composed of reference (plants and herbivore) and archaeological (herbivore) AA $\delta^{15}\text{N}$ values (for values see ESM 1; Table S1). Modern reference plant AA $\delta^{15}\text{N}$ values from Styring et al. (2014a) and Kendall et al. (2019) comprise those from cereal grains (barley and bread wheat), rachis (barley and bread wheat), and legume seeds (pea, broad bean) from controlled farms at Rothamsted (UK) and Bad Lauchstädt (Germany). Herbaceous (15 unique species) and woody (19 unique species) plants were gathered from Chew Valley (Somerset, UK), Bad Lauchstädt, and North Wyke Farm Platform (Devon, UK). Modern cattle AA $\delta^{15}\text{N}$ data from Kendall et al. (2017) provide individual AA trophic level offsets between plant diet and herbivore consumers. In addition, modern cattle act as a control for PCA and FRUITS outputs since these cattle grazed on a well-defined diet of C_3 grasses dominated by ryegrass (*Lolium perenne*). Archaeological samples from Styring et al. (2015) were gathered from domestic and wild herbivores from the Neolithic sites of Vaihingen an der Enz (Flomborn period), Makriyalos I, and Çatalhöyük East Mound. The archaeological herbivores selected from each site had similar bulk collagen N isotope values close to the mean $\delta^{15}\text{N}$ average for each site (ESM 3; Table S1).

AA $\delta^{15}\text{N}$ values were determined after successive steps of hydrolysis, purification through ion exchange chromatography, derivatization to *N*-acetyl-isopropyl esters. Derivatized AA $\delta^{15}\text{N}$ values were determined by gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS). Additional details are available in the relevant publications (Styring et al. 2014a; b; Styring et al. 2015; Kendall et al. 2017; Kendall et al. 2019).

Each data point used in the analysis is an average of triplicate measurements single point calibrated against a bracketing standard mixture of AAs with known $\delta^{15}\text{N}$ values. In order to compare herbivore $\delta^{15}\text{N}$ values directly with those of their diet, an AA-specific trophic adjustment was made to the herbivore bone collagen AA $\delta^{15}\text{N}$ values by subtracting the cattle diet trophic offsets determined by Kendall et al. 2017.

Statistical techniques

PCA was performed in Minitab release 17 (Minitab Inc.) to produce loading plots for the source diet categories of grain, rachis, woody and herbaceous plants. A loading plot is not provided for legumes due to the low number of samples ($n = 4$). Uncorrelated variables in the loading plots were used to produce a PCA score plot with data normalized to Glx (the averaged $\delta^{15}\text{N}$ signal of Glu and Gln) for plants, control cattle, and archaeological herbivores. Normalizing data for the score plot was necessary due to the amalgamation of data from manured and unmanured cereals; Styring et al. (2014b) showed that manured crops differed in absolute AA $\delta^{15}\text{N}$ values, but not in relative AA $\delta^{15}\text{N}$ values when compared to the unmanured crops.

Bayesian statistics were performed in FRUITS v3.0 (Fernandes et al. 2014) using normalized data. Since FRUITS is designed for use with bulk isotope data, some changes to the standard FRUITS configuration were made to accommodate CSIA-AA data. In addition to consumer and source AA isotope data, FRUITS

utilizes five parameters including proxies (consumer AA $\delta^{15}\text{N}$ values), sources (plant type), source fractions, weights, and concentrations. The AA $\delta^{15}\text{N}$ values of phenylalanine (Phe), proline (Pro), threonine (Thr), serine (Ser), and glycine (Gly) were selected from the loading plot to use as diet proxies because they were the least correlated variables (see Results and Fig. 1). Dietary sources are composed of grain, rachis, legumes, woody, and herbaceous plants. Source fractions (i.e., diet AA components) were based on all of the available AAs: alanine (Ala), valine (Val), leucine (Leu), Asx (averaged $\delta^{15}\text{N}$ values of deamidated asparagine and aspartic acid), Gly, Thr, Ser, Pro, and Phe, which were determined from the plants.

The weighting describes the contribution of N from the source AAs in different plants to the proxy AA $\delta^{15}\text{N}$ values derived from herbivores. For example, Phe is not biosynthesized, nor does it undergo reversible reaction with other AAs (Matthews 2007). Due to this, the source fraction Phe is weighted 100% to proxy Phe because the herbivore Phe signal is derived entirely from diet. Unlike Phe, the N in Pro is cycled between most other AAs via Glx because it is biosynthesized via a reversible ring closure from Glu (Bertolo and Burrin 2008). Therefore, the $\delta^{15}\text{N}$ value of Pro in bone collagen is likely an average of the source fraction trophic group AAs (e.g. the metabolic N pool described in O'Connell 2017). Therefore, Pro is equally weighted among all the contributing AAs. For the unique case of Thr, whose $\delta^{15}\text{N}$ value generally decreases with increasing trophic level (Wallace and Hedges 2016), a significant amount (more than 50%) of Thr is directly incorporated from diet (Van der Schoor et al. 2002). While Thr is not biosynthesized in mammals (Wallace and Hedges 2016), uncertainty surrounding Thr ^{15}N depletion and possibility of microbial biosynthesis (Metges 2000) led to conservative estimation of source fraction Thr contributes to proxy Thr as 50%. Gly and Ser has been found to be significantly ^{15}N -enriched in cattle (Kendall et al. 2017) and are thus treated as a trophic amino acid in the same way as Pro. Given that there are some considerable ambiguities surrounding the precise AA contribution from diet versus *de novo* synthesis (directly by the consumer or via rumen microbes unique to ungulates), which may also vary depending on nutrition, species, and other variables, 20% error is assumed on the input values (for more detail, see Electronic Supplementary Material 2, ESM 2; Table S1).

Concentration describes the original abundance of the AA in those same sources. The AA concentration of different plant groups were determined by calibration with Nle as an internal standard and then averaged together (for details on internal standardization, see Styring et al. 2015 and Kendall et al. 2019, for input values refer to ESM 2; Table S2). No priors were used here in order to best evaluate the method independently from bias.

The AA $\delta^{15}\text{N}$ values used as inputs for PCA and FRUITS are available in supplementary data (see ESM 1; Tables S1, S2, and S3 for PCA, ESM 2; Tables S1 and S2 for FRUITS).

Results

Principal component analysis (PCA)

PCA was performed in two parts. First, PCA was performed using unnormalized AA $\delta^{15}\text{N}$ values of unmanured crops, woody and herbaceous plants to explore correlation between AAs through loading plots (Fig. 1). The loading plots of woody and herbaceous floral AA $\delta^{15}\text{N}$ values showed that in general, Gly is less correlated with Glx than other AAs, with Ser falling between the two (Fig. 1). Thr is more correlated with Glx in woody plants but much less correlated in herbaceous plants. Pro is generally correlated with Glx but shows less correlation than the AAs Ala, Asx (combined N of aspartate and amino N of asparagine), Leu, and Val. The group of AAs that highly correlate with Glx in woody and herbaceous plants include Val, Leu, Asx, Pro, and Ala, which also corresponds with trophic AAs in mammals as defined by O'Connell (2017). Furthermore, Phe is also consistently correlated with Glx in plants. In general, herbaceous plants had similar loading distributions to woody plants (Fig. 1). The differences primarily reflect the closer correlation of Phe, Thr, and Ser with Glx in woody compared to herbaceous plants. These variables are therefore likely important in separating herbaceous and woody plants.

The cereal parts rachis and grain were also analyzed (Fig. 1). Due to the limited number of crop samples ($n = 8$ for grain and rachis), the AAs which consistently correlated with Glx in herbaceous and woody plants, i.e. Ala, Leu, and Val, were left out of the analysis. In rachis, Gly and Thr were least correlated with Glx, with Ser in between Gly and Glx. The remaining AAs Phe, Asx, and Pro were highly correlated with Glx. This pattern mirrored the AAs in herbaceous and woody plants. The AA correlations in grains were more unusual. Thr is uncorrelated with Glx as expected, but Gly and Ser were more correlated with Glx than in other plants, while Phe was less correlated with Glx than expected. Due to the unique AA correlations in grains, Gly, Ser and Phe can be expected to separate between grain and rachis, as well as herbaceous and woody plants. A loading plot for legumes is not available due to low sample count being insufficient for analysis ($n = 4$).

In order to reduce noise, only the most uncorrelated AAs in the loading plots (Fig. 1) were used to generate the plants-only score plot (Fig. 2a). The score plot shown was therefore built with the $\delta^{15}\text{N}$ values of Phe, Thr, Pro, Ser, and Gly. Normalizing values to Glx, i.e. $\delta^{15}\text{N}_{\text{Glx}} - \delta^{15}\text{N}_{\text{AA}}$, to remove the effect of environmental variables (e.g. manuring, wetland N enrichment) zeroed the Glx values; therefore, Glx was omitted as a variable. Instead Pro, which is well correlated with Glx in plants (Fig. 1) and in herbivores (Kendall et al. 2017), acts as a substitute. The legumes formed their own individual cluster with the two samples of pea nearly overlapping and separated from the two samples of broad bean (Fig. 2a). Rachis and grain also formed unique clusters. The herbaceous plants are generally separated from woody plants, but there is some overlap between the two categories, likely due to the considerable variety of species sampled (19 unique woody and 15 unique herbaceous species). Furthermore, the crops are shown to be more related to the herbaceous than the woody plants in PC1, with rachis being more closely related to herbaceous and woody plants than the cereal grains and legumes. Finally, the control cattle firmly overlapped with herbaceous plants as expected (Fig. 2b), which demonstrates the applicability of PCA for dietary reconstruction.

Food Reconstruction Using Isotopic Transferred Signals (FRUITS)

Phe, Thr, Pro, Ser, and Gly were used as proxies in the FRUITS model to be comparable with PCA results. The $\delta^{15}\text{N}$ values of these AA from the modern cattle (n = 4) grazing on controlled pastures comprising 100% unmanured C₃ herbs were used to evaluate the accuracy of FRUITS predictions of diet. The FRUITS model predicted that herbaceous plants dominated, comprising 33 ± 21% of diet, while woody plants made up the second most important contribution at 24 ± 19% (Table 1). Grain, rachis, and legumes were predicted to have contributed 10 – 20% of diet. The results clearly demonstrate some discrepancy between known diet and model predictions.

Table 1

FRUITS output for percent contribution (%) of the source groups with one standard deviation in brackets. Control cattle is included as comparison to archaeological herbivores.

Herbivore*	N	Grain	Rachis	Legume	Herbaceous	Woody	Crop	Non-crop
CH	5	15.8	15.0	22.67	26.39	20.14	53	47
B.primigenius		(13.55)	(13.26)	(16.47)	(18.58)	(16.61)		
CH	5	15.39	15.14	21.47	27.0	21.0	52	48
ovicaprid		(13.43)	(13.12)	(15.8)	(19.13)	(17.06)		
MAK	5	15.08	14.03	14.95	30.66	25.28	44	56
Ovis sp.		(13.31)	(12.92)	(12.44)	(20.95)	(19.71)		
MAK	2	19.35	17.44	16.87	25.22	21.13	54	46
C. elaphus		(15.67)	(14.81)	(13.52)	(18.23)	(16.87)		
MAK	4	17.59	15.95	19.05	26.93	20.48	53	47
B. taurus		(14.69)	(13.72)	(14.83)	(18.8)	(16.73)		
VAH	5	17.56	16.01	21.46	25.71	19.27	55	45
B. taurus		(14.79)	(13.86)	(16.2)	(18.81)	(16.15)		
VAH	5	18.68	16.43	20.83	24.95	19.12	56	44
C. elaphus		(15.33)	(14.16)	(16.25)	(18.15)	(15.96)		
Control	4	12.05	12.7	18.51	32.58	24.16	43	57
B. taurus		(10.86)	(11.4)	(13.58)	(20.93)	(18.73)		
* CH, MAK, and VAH refer to Çatalhöyük, Makriyalos, and Vaihingen respectively.								

Case studies

For each archaeological site, a score plot was generated, comparing the normalized $\delta^{15}\text{N}$ values of Phe, Thr, Pro, Ser, and Gly from herbivore bone collagen with those of the modern reference plants. The faunal AA $\delta^{15}\text{N}$ values were made comparable to those of the plants by using an AA-specific trophic adjustment, as determined by Kendall et al. (2017) (see Materials and Methods). A corresponding FRUITS analysis of predicted herbivore diet for each species and site was also carried out. Since not all AAs are represented, loading plots were excluded, but are available as supplementary materials (ESM 3; Fig. S1).

Çatalhöyük

For Çatalhöyük, the wild herbivores considered in this analysis are represented by a single species, aurochs (*Bos primigenius*, $n = 5$), while the domestic herbivores comprised individuals belonging to the *Ovis* and *Capra* genera (ovicaprids, $n = 5$). Both groups appeared to be more closely related to the herbaceous than woody plants in the score plot (Fig. 3a). The domestic herbivores appear to be slightly separated from the wild cattle on PC1, but there is some overlap.

Makriyalos

At Makriyalos, the wild herbivores considered here are represented by red deer (*Cervus elaphus*, $n = 2$), while the domestic herbivores consisted of sheep (*Ovis sp.*, $n = 5$) and cattle (*Bos taurus*, $n = 4$). The domestic herbivores cluster tightly together without discrimination between cattle and sheep (Fig. 3b) and overlap with herbaceous plants. There is clear separation of the domestic and wild herbivores on PC2. It is possible that the two wild red deer had some dietary contribution from woody plant or rachis sources due to a slightly closer association with these groups.

Vaihingen an der Enz

At Vaihingen, the wild herbivores in the study are represented by red deer ($n = 5$), while the domestic herbivores consisted of cattle ($n = 5$). Both the domestic and wild herbivore groups show similar degrees of dispersion and appear to overlap most closely with herbaceous plants (Fig. 3c).

FRUITS predictions for archaeological herbivore diet

Uncertainties for the predicted diets of archaeological herbivores are large (Table 1). The uncertainties associated with the predicted contributions of herbaceous and woody plants were particularly large compared to those of the crops, which is expected due to the large number of different species represented within non-crops. Increased variance associated with herbaceous and woody plants is also reflected in the raw data and the PCA plots (Fig. 2a). In all cases, crops of each category are predicted to have contributed no more than 20% of the total diet.

Table 1 shows estimates for the dietary contribution from crop (grains, rachis, legume seeds) and non-crop (herbaceous plants, woody plants) sources. In all cases, herbaceous plants were estimated to have been the most important diet component followed by woody or legume plants. With the exception of red deer at Makriyalos, grain and rachis were estimated to be the least important dietary component.

Furthermore, total diet contribution from crops were combined and compared against contribution from non-crops. Most herbivores were found to have higher total diet contribution from crops rather than non-crops (6–12%), except sheep at Makriyalos (-12%) and modern cattle fed on controlled pastures (-14%). Limitations of FRUITS results are further elaborated in the discussion.

Discussion

The animals originally selected for CSIA-AA from each site had similar bulk collagen N isotope values close to the mean average for each site (ESM 3; Table S1). Consequently, the results of this study must be interpreted as the likely diet of a particular group of animals from each site, which probably had access to similar plant resources and, in the case of the domestic herbivores, had been managed in similar ways to others in the assemblage.

Exploring the relationships between AA $\delta^{15}\text{N}$ values in modern reference plants

Nitrogen is incorporated into plants via the GS-GOGAT pathway as Gln and Glu (Miflin and Lea 1977), which was confirmed by ^{15}N -labelling experiments, in which plants show the greatest amount of ^{15}N uptake in these AAs (Yoneyama et al. 2003). Since Glx is central to plant AA metabolism due to providing amino groups to the other AAs (Forde and Lea 2007), it is to be expected that most AA $\delta^{15}\text{N}$ values would be highly correlated with that of Glx, which is found to be the case for Ala, Asx, Leu, Pro, Val and Phe (Fig. 1). A decreased degree of correlation between an AA and Glx is likely only to result when there are many intermediate steps to biosynthesis or when there are partially self-sustaining recycling mechanisms.

Gly and Ser are involved in one such recycling pathway. The SHMT-SGT pathway uses two molecules of Gly to produce one molecule of Ser. Excess Ser is in turn recycled into molecules of Gly. This pathway produces a deficit of one molecule of Gly if Ser is recycled from excess Gly. The Gly deficit can be balanced by biosynthesis from Glu (Bauwe et al. 2010). This is likely to explain why Gly and Ser can be less correlated with Glx than many of the other AAs (Fig. 1). Previous studies have indicated that increased biosynthesis of lignin via the phenylpropanoid pathway can lead to elevated Phe $\delta^{15}\text{N}$ values (Kendall et al. 2019). This phenomenon has been suggested to be a distinguishing factor between cereal grains and rachis as well as herbaceous and woody plants, since rachis and woody plants contain more lignin than grains and herbaceous plants (Kendall et al. 2019). In Fig. 1, Phe is less correlated with Glx in herbaceous plants compared with woody plants. Increased demand for Phe in woody plants for lignin production would lead to higher levels of Phe biosynthesis from Glu, thereby increasing correlation with Glx. Interestingly, the consistent correlation of Phe with Glx in plants (Fig. 1) could explain the apparent stability of the $\Delta^{15}\text{N}_{\text{Glx-Phe}}$ value (Steffan et al. 2013) commonly used to quantify trophic level differences (McMahon and McCarthy 2016). The implication is that if the correlation between Phe and Glx weakens, such as in the case with herbaceous plants or cereal grains, the $\Delta^{15}\text{N}_{\text{Glx-Phe}}$ value could become less reliable. Finally, in plants, Thr is biosynthesized from Asp without breaking of a C – N bond. Lack of consistent correlation of Thr with Asx (Fig. 1) could be explained due to conversion of Thr to Gly

through threonine aldolase (Joshi et al. 2006), the activity of which may be highly variable between species. Our loading plots of modern reference plant AA $\delta^{15}\text{N}$ values (Fig. 1) is therefore consistent with known AA metabolic pathways and validates the differences in metabolism between various different plant categories under analysis.

These differences in AA metabolism between the different plant categories means that they are reasonably well-separated in a score plot (Fig. 2a). While a loading plot could not be generated for the legumes, the patterning of their AA $\delta^{15}\text{N}$ values differs significantly from that of the other plant categories (ESM 1; Table S1, Styring et al. 2014a), allowing them to be separated from the other plants in Fig. 2a.

Assessing the potential and current limitations of using AA $\delta^{15}\text{N}$ values and FRUITS to reconstruct herbivore diet

Modern reference cattle overlap with herbaceous plants in Fig. 2b as expected in the score plot. However, while the overlap demonstrates a strong relationship between herbaceous plants and consumer diet, FRUITS was utilized to provide a quantitative estimate of percentage contribution from various dietary sources. The known diet of the control cattle from North Wyke Farm Platform was 100% C_3 herbaceous plants, which is not what the FRUITS model predicts (Table 1).

Given the evident limitation in applying the FRUITS model, a combined percent contribution of crops (grain, rachis, legume) and non-crops (herbaceous and woody plants) was calculated in Table 1. The score plot (Fig. 2a) shows sufficient separation of crops and non-crops, which justifies this separate categorization. In the case of control cattle, this leads to a predicted 57% contribution from non-crop sources and 43% from crops. A 57% contribution of non-crops to the diet remains significantly lower than the actual contribution of 100%. This error can be attributed to the large standard deviations in the source data (as illustrated by data dispersion in Fig. 2a). Moreover, given the large uncertainties, it is unsurprising that there is insufficient statistical certainty to assign 0% contribution to crops. If some value (such as 10%) must be assigned to a category due to statistical uncertainty, even when the real contribution is 0%, then this error implies overestimation of crop contribution in Table 1, given that there are three categories of crops and only two of non-crops. Since the same source plant values were used when analyzing the unknown data, the predicted diets of control cattle outline the limits to which FRUITS results can be interpreted. Moreover, applying FRUITS to CSIA-AA data necessitates estimating percent contribution of different source AA amino groups to biosynthesized AAs found in bone collagen. Without a greater understanding of AA cycling through the 'metabolic pool' (O'Connell 2017), which may vary between species and can be influenced by rumen microbes, it is difficult to achieve more precision. While uncertainty can be reduced with priors in Bayesian models, use of bad priors can have significant deleterious consequences for decreasing model accuracy, while giving the appearance of increasing precision (Cheung and Szpak 2020). Given these limitations and the relative performance of PCA and FRUITS in predicting the diet of control cattle, PCA results should be emphasized over FRUITS.

Using CSIA-AA to reconstruct herbivore diet at three Neolithic archaeological sites

Çatalhöyük

The overlap of domestic and wild herbivores with herbaceous plants in the score plot (Fig. 3a) is consistent with current interpretations of animal management strategies at Çatalhöyük. Current evidence suggests that the Konya plain surrounding Çatalhöyük was a dryland anabranching river system in the Neolithic (Ayala et al. 2017) and strontium isotope analysis of sheep tooth enamel suggests that sheep were likely to have been herded on this plain rather than in the more distant upland region (Bogaard et al. 2014). Aurochs, like their domestic counterpart, are primarily grazers (Gordon and Prins 2008). Therefore, in a region such as the Konya plain, it is reasonable to assume that herbaceous plants would have been the dominant dietary component. Likewise, the C and N isotope values of sheep vary greatly ($\delta^{13}\text{C}$ range 7.4‰, $\delta^{15}\text{N}$ range 9.5‰, $n = 235$), which Pearson et al. (2007, 2021) suggests reflects herding in multiple isotopically distinct environments rather than as a result of feeding on crops used as fodder. Moreover, recovered archaeological goat/sheep dung pellets also reflect high dietary variance with weed species from wetlands (*Bolboschoenus glaucus*, *Carex*), common weeds (e.g. *Chenopodium album*, *Polygonum aviculare*), and C_4 species likely derived from saline marshes (*Aeluropus*, *Crypsis*, *Sporobolus*) (Charles et al. 2014). The results in the score plot (Fig. 3a) corroborate these pre-existing sources of evidence.

Furthermore, the bulk collagen $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values of ovicaprids ($\delta^{13}\text{C}$: $-19.4 \pm 0.9\text{‰}$, $\delta^{15}\text{N}$: $5.2 \pm 0.1\text{‰}$, $n = 5$) and aurochs ($\delta^{13}\text{C}$: $-18.7 \pm 0.8\text{‰}$, $\delta^{15}\text{N}$: $9.7 \pm 1.4\text{‰}$, $n = 5$) overlap with each other, which further supports the interpretation suggested by our modeling that domestic herbivores were herded in a similar environment to their wild counterparts (for a complete list of bulk collagen values see ESM 3; Table S1).

Makriyalos

The domestic and wild herbivores from Makriyalos form two distinct groups in the score plot (Fig. 3b). This might suggest tightly controlled herding of domestic herbivores at Makriyalos, meaning that they had access to plants that were distinct from those available to the red deer. The domestic herbivores overlap mostly with herbaceous plants, while the red deer plot closer to woody plants and rachis. These results support the possibility of herding of domestic herbivores in local saline marshes, as suggested in Vaiglova et al. (2018). The more mixed diets of the two red deer individuals are consistent with known feeding patterns of red deer. Contrary to most deer species, the red deer is not predominantly a browser. Red deer obtain as much as a third of their diet from grasses with the rest made up of concentrate foods which include items such as leaves, shrubs, and seeds (Gebert and Verheyden-Tixier 2001). In regions with sparse woodlands such as the plains surrounding Makriyalos, grasses and herbs can make up a significant portion of diet, so a large herbaceous plant contribution to red deer diet is not unexpected.

Vaihingen an der Enz

There is no distinct separation of domestic and wild herbivores in the score plot for Vaihingen (Fig. 3c), and both overlap with herbaceous plants. The red deer did not overlap with the woody plants as might be expected. Instead, red deer appear to have had a similar diet to the domestic fauna. This interpretation agrees with bulk C and N isotope values. Despite relatively high cereal grain $\delta^{15}\text{N}$ values (Fraser et al. 2013), cattle $\delta^{15}\text{N}$ values ($6.8 \pm 0.2\text{‰}$, $n = 5$) are low and not significantly different from red deer ($6.2 \pm 0.1\text{‰}$, $n = 5$), which does not suggest crop foddering. Cattle $\delta^{13}\text{C}$ values ($-22.7 \pm 0.5\text{‰}$) are also similar to those of red deer ($-23.2 \pm 0.3\text{‰}$) and are noticeably more depleted (average difference approximately 4‰) compared to herbivores from the other sites, which is likely a result of the canopy effect in wooded environments (see ESM 3; Table S1). These results suggest that cattle and red deer fed in similarly wooded environments.

The similarity between wild and domestic herbivores can be explained either by cattle grazing in a similar wooded environment to deer or through some degree of leaf foddering as has been suggested for herbivores in Switzerland (Doppler et al. 2017) and France (Balasse et al. 2012). Deer and cattle investigated in this study have lower $\delta^{13}\text{C}$ values than sheep ($-21.8 \pm 1.7\text{‰}$, $n = 4$), goats ($-21.1 \pm 0.3\text{‰}$, $n = 2$), and domestic pigs ($-21.3 \pm 0.3\text{‰}$, $n = 23$) from the same site (Fraser et al. 2013). This further supports evidence of some woodland browsing or leaf foddering but from isotopic dietary reconstruction alone, it cannot be determined if lower $\delta^{13}\text{C}$ values are due to leaf foddering or herding in woodland environments.

Complications associated with C_4 plant consumption

While this model can separate out clusters of C_3 plants with distinctive metabolisms, a significant limitation remains due to the lack of C_4 plants in the source data. When compared with C_3 plants, C_4 plants have somewhat elevated bulk $\delta^{15}\text{N}$ values (by ca. 1.5‰) in addition to elevated $\delta^{13}\text{C}$ values (Hare et al. 1991). Furthermore, Hare et al. (1991) showed that the $\delta^{15}\text{N}$ values of the AAs Gly and Ser are significantly higher in C_4 plants compared to C_3 plants ($\delta^{15}\text{N}$ values were elevated by 3.2‰ and 3.7‰ respectively for Gly and Ser in C_4 plants). The reference plants in this study are all C_3 and the trophic offset between plant diet and herbivore consumer was determined with C_3 plants (Kendall et al. 2017), which given $\delta^{15}\text{N}$ value differences between C_3 and C_4 plants, is not necessarily directly applicable to herbivores fed with C_4 grasses.

These metabolic differences between C_3 and C_4 plants are encouraging for future studies; however, at the present, it means that for sites with evidence of C_4 plant consumption, the models may not be accurate. At Çatalhöyük, it is possible that some C_4 plant contribution could have skewed the FRUITS results, as relatively high $\delta^{13}\text{C}$ values of both wild cattle and domestic ovicaprid bone collagen indicate a dietary contribution of C_4 plants (Pearson et al. 2015) and seeds of C_4 grasses have been found in sheep dung pellets (Charles et al. 2014). Likewise, herding in saline marshes is also a possibility for sheep and particularly cattle at Makriyalos. The average bulk $\delta^{13}\text{C}$ value of $-19.4 \pm 0.9\text{‰}$ (ESM 3; Table S1) for

sheep ($n = 5$) indicates that they could have had a mixed C_3/C_4 diet while cattle at Makriyalos show elevated $\delta^{13}C$ values averaging at $-18.0 \pm 2.1\text{‰}$, with the median at -16.7‰ for individuals analyzed in the score plot ($n = 4$) (ESM 3; Table S1). More representative sampling of cattle concludes with similar findings (Vaiglova et al. 2018). Therefore, reference C_4 plants and unique saline marsh species should be sampled to further augment the model.

Conclusion

The case studies referenced in this paper are restricted by use of historical data, but these results demonstrate that multivariate approaches using many AAs can allow for better visualization and extraction of information from CSIA-AA data than relying solely on raw values or the $\Delta^{15}N_{\text{Glx-Phe}}$ value alone. However, despite success with using PCA, Bayesian modeling through FRUITS still faces significant challenges. Lack of C_4 reference plants likely contributes to some of the large degree of uncertainty in archaeological herbivore diet. However, even with well characterized control cattle fed on C_3 herbs, FRUITS predictions still display large uncertainties. With these limitations in mind, multivariate analysis of AA $d^{15}N$ values from herbivores at Çatalhöyük, Makriyalos, and Vaihingen returned results which support current site interpretations and further advance the idea of distinctive herding practices at Makriyalos as suggested in Vaiglova et al. (2018). Future work should focus on refining the models by remedying gaps in existing knowledge of C_4 plant nitrogen metabolism and its relationship to herbivore AA $\delta^{15}N$ values, as well as considering alternative methods of quantifying the dietary contribution of plant types until robust priors become available for Bayesian analysis.

Declarations

Acknowledgements: We thank Rothamsted Research and the Lawes Trust for access to the archived Rothamsted crop samples. We also thank Ines Merbach for sample collection from the Bad Lauchstädt Experimental Research Station, and the late Robert Orr for assistance with sample collection from the North Wyke Farm Platform, a UK National Capability supported by the Biotechnology and Biological Sciences Research Council (BBSRC BB/J004308/1).

Funding: The AA $d^{15}N$ values of modern crops and archaeological herbivore bone collagen were determined whilst AKS was in receipt of a NERC studentship (RE2158) that formed part of NERC standard Grant NE/E003761/1 (PI Bogaard). The AA $d^{15}N$ values of modern herbaceous and woody plants and cattle bone collagen were determined whilst IK was funded by the European Research Council (ERC) Advanced Grant NeoMilk (FP7-IDEAS-ERC/324202, to RPE). NERC (Reference: CC010) and NEIF (www.isotopesuk.org) are thanked for funding and maintenance of the GC-C-IRMS instruments used for this work.

Conflict of Interest: The authors declare that they have no conflict of interest.

Ethical approval: For this type of study formal consent is not required.

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Figures

Fig. 1

Figure 1

Loading plots showing correlation between AA $\delta^{15}\text{N}$ values for unnormalized plants. The amount of variance explained by principal component (PC) is reported for PC1 vs. PC2 for a herbaceous plants (n = 26) and b woody plants (n = 27). Highly correlated AA $\delta^{15}\text{N}$ values Leu, Ala, and Val in plots a and b are not analyzed for c grains (n = 8) and d rachis (n = 8).

Fig. 2

Figure 2

Score plots of modern plants and herbivores normalized to Glx. Plot a shows separation of grain (n = 8), rachis (n = 8), legumes (n = 4), woody plants (n = 27), and herbaceous plants (n = 26). In plot b, modern cattle (n = 4) fed on fields of *Lolium perenne* is tropically adjusted, normalized, and analyzed with modern plant materials. Plant data was plotted irrespective of fertilization conditions.

Fig. 3

Figure 3

Score plots of trophic adjusted and normalized domesticated and wild archaeological herbivores analyzed with experimental plants at three Neolithic sites. The site of a Çatalhöyük includes domestic ovicaprids ($n = 5$) and wild herbivore *Bos primigenius* ($n = 5$). b Makriyalos includes domesticated sheep *Ovis aries* ($n = 5$), domesticated cattle *Bos taurus* ($n = 4$), and wild red deer *Cervus elaphus* ($n = 2$). c Vaihingen includes domesticated cattle ($n = 5$) and wild red deer ($n = 5$).

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