Reconstructing Hominin Diets with Stable Isotope Analysis of Amino Acids: New Perspectives and Future Directions

THOMAS LARSEN®, RICARDO FERNANDES®, YIMING V. WANG®, AND PATRICK ROBERTS®

Stable isotope analysis of teeth and bones is regularly applied by archeologists and paleoanthropologists seeking to reconstruct diets, ecologies, and environments of past hominin populations. Moving beyond the now prevalent study of stable isotope ratios from bulk materials, researchers are increasingly turning to stable isotope ratios of individual amino acids to obtain more detailed and robust insights into trophic level and resource use. In the present article, we provide a guide on how to best use amino acid stable isotope ratios to determine hominin dietary behaviors and ecologies, past and present. We highlight existing uncertainties of interpretation and the methodological developments required to ensure good practice. In doing so, we hope to make this promising approach more broadly accessible to researchers at a variety of career stages and from a variety of methodological and academic backgrounds who seek to delve into new depths in the study of dietary composition.

Keywords: Isotope fingerprinting, trophic ecology, human nutrition, archeology, paleoecology

Investigating hominin diets and environments has long been at the forefront of paleoanthropological and archeological research. In deep time contexts, there have been major debates as to the degree to which increasing meat consumption and procurement in more open environments set the hominin clade apart from the lineages of our closest extant great ape relatives (e.g., Dominguez-Rodrigo et al. 2014). Physiologically, the ability to target nutritionally dense foods and process (e.g., through cooking) dietary resources has been linked to hominin trajectories of expanding brains, lengthening of small intestines, and shrinking large intestines (Armelagos 2014). Meanwhile, in the context of our own species’s evolution in Africa approximately 300,000 years ago and expansion around much of the globe by the end of the Late Pleistocene, it has been proposed that Homo sapiens demonstrates a dietary flexibility and adaptiveness not seen among other hominins (Roberts and Stewart 2018). Investigations of Holocene humans have been crucial for exploring the potential social, economic, and political ramifications of the emergence of food production (Richards et al. 2003, Jones and Rowley-Conwy 2016) and, later, urbanism (Twiss 2012, Styring et al. 2017) in the archeological record. Certainly, in ethnographic, historical, and archeological contexts, the processing, consumption, and procurement of food have persistently played a major role in cultural expression, political demarcation, economic organization, and adaptations to changing climates and environments (e.g., Forson and Counihan 2013), all issues that remain important topics in a dynamic twenty-first century world. The detailed investigation of hominin nutrition, dietary change, and variabilities in food sources across space and time therefore has much to tell us about where we come from and where we are going.

Much of what we know about past hominin nutrition, physiology, and diet comes from observational studies of modern populations and physical examination of skeletal remains and artifacts from burials and excavation sites (Ungar et al. 2006, Reed 2021). In particular, the analysis of the organic remnants of plants and animals can provide detailed snapshots into what past hominins might have been eating (Hedges 2009), as well as the ecosystems in which they might have lived (Beuning et al. 2011). From the 1970s onward, however, stable isotope analysis of bulk organic and inorganic hominin tissues emerged as an important means of directly providing broad overall perspectives on how much of different food sources individuals might have been...
consuming (Van Der Merwe and Vogel 1978, Lee-Thorp et al. 1989), often even yielding dietary information in contexts in which the remains of food sources may not themselves be preserved (Pate 1994). The stable isotope ratios of bulk nitrogen ($\delta^{15}$N) are usually used to infer trophic position as the enrichment of $^{15}$N of the total proteinaceous tissue is generally 3‰–5‰ over that of the diet (Smith and Epstein 1971). By contrast, the stable isotope ratio of bulk carbon ($\delta^{13}$C) has been primarily used as an ecological source tracer because $^{13}$C enrichment during trophic transfer is small, usually less than 1‰, but $\delta^{13}$C varies predictably among different plant groups (e.g., C$_4$ and C$_3$) and environmental settings (van der Merwe and Medina 1991). Nevertheless, during the last two decades, it has become increasingly clear that dietary reconstructions based on bulk stable isotope ratios are hindered by two major complicating factors. First, numerous food sources can have similar $\delta^{13}$C and $\delta^{15}$N values, limiting the resolution of interpretation. Second, the isotopic baselines (i.e., $\delta^{13}$C and $\delta^{15}$N values for food sources at the base of an ecosystem) can vary significantly with location, season, and environment. For example, the $\delta^{15}$N range between nitrogen-fixing plants and those fertilized by seabird guano can be as large as 20‰ in plant tissues (Szpak et al. 2014), and it is not unusual that the $\delta^{13}$C range of algal biomass produced during low and high productivity periods is 10‰ (Guiry 2019). In paleodiets, it can be particularly challenging to obtain this baseline information and therefore reliably and accurately interpret hominin dietary choices.

The three main determinants of stable isotope ratios in consumer tissues are diet; digestive processes; and, for stable carbon isotopes, the dietary routing of carbohydrates, protein, and lipids to tissue synthesis (Fernandes et al. 2012, Hobbie 2017). Although it may not be feasible in an archaeological context, accurate interpretation of bulk stable isotope ratio data from collagen, the main organic constituent in bones, is based on knowledge of the composition and isotopic values of dietary macronutrients. The isotopic values are often estimated from measurements of food remains (e.g., animal bone collagen, charred plants), although the main edible fractions may not be available for analysis. In this case, estimates of the isotopic values of the edible fractions are made from known offsets between the measured fractions in archeological samples (e.g., bone collagen versus muscles; Fernandes et al. 2015, Bownes et al. 2017, Soncin et al. 2021). As for macronutrient food composition, modern composition values are sometimes employed, although these can vary as a result of several factors (e.g., the fat content of consumed flesh from different animal species may depend on catch season and on the selection of cuts and organs for consumption, the effects of cooking). An alternative approach to estimating macronutrient contributions for different food types relies on data for average human consumption patterns (Fernandes et al. 2015). Dietary estimates can be obtained using mixing models that incorporate multiple parameters (food macronutrient content and isotopic values, dietary routing, and consumer-to-diet isotopic offsets; Fernandes et al. 2015, Cheung and Szpak 2020). However, their estimate precision may be limited, given the compounded uncertainties in these parameters.

To overcome food source equifinality, baseline issues, and the uncertainties regarding dietary routing associated with bulk stable isotope ratios, archeologists are increasingly using amino acid stable isotope ratios. These nitrogenous molecules are key for nutrient exchange, because every living organism synthesizes its proteins from the same set of 20 amino acids. They are also used to form other biomolecules or are oxidized to urea and carbon dioxide as a source of energy. Pioneering work three decades ago on both modern and fossil proteins recognized that distinct metabolic processes control isotope patterns among individual amino acids in consumers and their food sources (Edgar Hare et al. 1991). However, the great potential of using these patterns to determine dietary variability only emerged in the following decade from collective evidence gathered across multiple fields. Amino acids can be functionally divided into two groups on the basis of their carbon skeletons (Borman et al. 1946, Womack and Rose 1947, Reeds 2000). The first group, the essential amino acids, cannot be synthesized in the body and must therefore be provided by the organism’s diet. The second group, the nonessential amino acids, either can be provided directly by the diet or can be synthesized in the body from metabolic intermediates derived from dietary fat, carbohydrates, and proteins (box 1). Despite the organism’s ability to de novo synthesize nonessential amino acid carbon skeletons, it is not always achieved at a rate that meets metabolic demand and may become limiting for growth and vital metabolic functions (Womack and Rose 1947, Horvath et al. 1996). A separate set of metabolic processes control the fate of amino acid nitrogen (box 1). Trophic amino acids readily transaminate and exchange nitrogen with other amino acids during trophic transfer in contrast with the source amino acids that largely retain their $\delta^{15}$N amino acid values. Since these metabolic processes cause a much greater $^{15}$N enrichment in the trophic than source amino acids, researchers are increasingly using paired isotopic spacing as means of inferring trophic position while circumventing the issue of varying isotopic baselines (McClelland and Montoya 2002, Popp et al. 2007, Yoshito et al. 2007, Ohkouchi et al. 2017).

Archeologists, often in collaboration with biochemists and ecologists, have contributed with insights about the controls of amino acid stable isotope ratio patterns via controlled feeding studies and phenomenological studies (Edgar Hare et al. 1991, Fogel and Tuross 1999, 2003, Corr et al. 2005, Honch et al. 2012, Jarman et al. 2017, Webb et al. 2017). Increasingly, amino acid stable isotope ratio analysis has also been applied to varying archeological and paleoecological contexts, from Neanderthal dietary preferences and complexity relative to Homo sapiens (Naito et al. 2016a) to the diets of Polynesian populations living on the supposedly ecologically vulnerable island of Rapa Nui (Easter Island; Jarman et al. 2017).
Box 1. Metabolism of amino acid nitrogen and carbon follows distinct pathways.

The schematic representations of amino acid metabolic pathways in figure 1 are depicted in the liver because it is the body's main hub for amino acid metabolism and protein synthesis, degradation and detoxification. Exceptions include the branched chain amino acids (leucine, isoleucine, valine), where the initial catabolic step takes place in extrahepatic tissues (i.e., tissues beyond the liver). Pertaining to nitrogen, the amino nitrogen is transaminated to ammonia. The excess ammonia is converted into urea in the liver through the urea cycle, and the remaining ammonia becomes part of the metabolic amino nitrogen pool. Those amino acids whose nitrogen can be readily exchanged in a single chemical step, the trophic amino acids, most likely share this metabolic amino nitrogen pool with other trophic amino acids (O’Connell 2017). The source amino acids do contribute to the common amino nitrogen via irreversible transamination. An anomaly to the trophic and source amino acids is threonine, which is denoted as a metabolic amino acid, because it is $^{15}$N depleted during trophic transfer (Edgar Hare et al. 1991).

Pertaining to the carbon skeletons, the nonessential amino acids can be grouped according to their association with biosynthetic pathways. The glycolytic amino acids are synthesized from metabolic intermediates (3-phosphoglyceric acid, phosphoenolpyruvic acid) of the glycolytic pathway (in the cytosol) and the tricarboxylic acid cycle (α-Ketoglutaric acid, oxaloacetate) amino acids are synthesized from intermediates of the Krebs cycle (in the mitochondria). Glucose and glycerol are sourced to the glycolytic pathway, and fatty acids and short-chain fatty acids are sourced to the tricarboxylic acid cycle. Tricarboxylic acid products can also function as intermediates for Ala via routing to phosphoenolpyruvate and pyruvate. The catabolism of excess amino acids either occurs via gluconeogenesis or ketogenesis. Gluconeogenesis is the synthesis of glucose from noncarbohydrate precursors such as the glucogenic amino acids (marked with a 1) and ketogenesis is the metabolic pathway for producing ketone bodies by breaking down fatty acids and ketogenic amino acids (marked with a 2). A large group of amino acids can be catabolized by both processes (marked with a 3). The broken lines indicate transportation of metabolites across the cell membrane. Images from macrovector (http://freepik.com) under Creative Commons license.

Figure 1. Metabolism of amino acid nitrogen and carbon follows distinct pathways.

Pertaining to the carbon skeletons, the nonessential amino acids can be grouped according to their association with biosynthetic pathways. The glycolytic amino acids are synthesized from metabolic intermediates (3-phosphoglyceric acid, phosphoenolpyruvic acid) of the glycolytic pathway (in the cytosol) and the tricarboxylic acid cycle (α-Ketoglutaric acid, oxaloacetate) amino acids are synthesized from intermediates of the Krebs cycle (in the mitochondria). Glucose and glycerol are sourced to the glycolytic pathway, and fatty acids and short-chain fatty acids are sourced to the tricarboxylic acid cycle. Tricarboxylic acid products can also function as intermediates for Ala via routing to phosphoenolpyruvate and pyruvate. The catabolism of excess amino acids either occurs via gluconeogenesis or ketogenesis. Gluconeogenesis is the synthesis of glucose from noncarbohydrate precursors such as the glucogenic amino acids (marked with a 1) and ketogenesis is the metabolic pathway for producing ketone bodies by breaking down fatty acids and ketogenic amino acids (marked with a 2). A large group of amino acids can be catabolized by both processes (marked with a 3). The broken lines indicate transportation of metabolites across the cell membrane. Images from macrovector (http://freepik.com) under Creative Commons license.
et al. 2017, Commendador et al. 2019). Although such studies hold much potential and although these methodologies have been well reviewed for ecological (Nielsen et al. 2018, McMahon and Newsome 2019, Whiteman et al. 2019)—and, to a lesser extent, archeological—contexts (O’Connell and Collins 2018), they have often remained something of a black box for the wider archeological and anthropological communities, with the potential and limitations of amino acid stable isotope ratios in reconstructing past hominin diets and ecologies often remaining obscured. Meanwhile, ecologists and biochemists applying methods to archeological contexts might be unfamiliar with the particular taphonomic and interpretive issues of deep time hominin diets. In the present article, we highlight the utility of amino acid stable isotope ratios for deep investigations of hominin diets across time and space and discuss the next steps required for an expanded use of amino acid stable isotope ratios in archeology, paleoanthropology, and paleoecology. We will first outline how human digestive physiology and metabolic pathways affect amino acid stable isotope ratio patterns and then showcase δ¹⁵N氨基酸 amino acid and δ³⁴C氨基酸 amino acid applications via case studies and compiled literature data. In particular, we seek to demonstrate how amino acid stable isotope ratio patterns remain diagnostic of trophic position and food source, regardless of varying isotope baselines, but also how the potential of integrating baseline information with multivariate amino acid stable isotope ratio analysis can provide more robust insights into food sources. We propose a series of best practices for obtaining reliable amino acid stable isotope ratio results and for the statistical analysis of multivariate isotope tracer data. We also outline a series of perspectives and possibilities relating to this methodology moving forward. In doing so, we seek to make these approaches and their potential and pitfalls in the context of hominin diet and paleoecological reconstruction more accessible to researchers bridging the fields of ecology and anthropology.

Factors affecting isotope values
Beyond dietary practices, food processing, digestive and metabolic processes can affect amino acid stable isotope ratio values in major ways.

Food processing. Cooking can be construed as a form of external predigestion that lowers the concentration of certain antinutrients and the overall protein quality (Candela et al. 1997, Chau et al. 1997). No recent human populations are known to have lived without cooking, and it likely played a major role in influencing hominin diets and metabolisms from the Pliocene–Pleistocene onward (Wrangham and Conklin-Brittain 2003, Zink and Lieberman 2016). The ability to increase the nutritional value of plant- and animal-derived foods and access difficult to digest nutrients has opened up metabolic energy for other aspects of hominin biology, including brain growth (Aiello and Wheeler 1995), although this remains disputed (Cornélio et al. 2016). This is one reason the advent of cooking and the control of fire in the archeological and paleoanthropological records have received so much attention (Attwell et al. 2015, Smith et al. 2015, Wrangham 2017).

Poorly digestible animal proteins, such as collagen, elastin, and keratin, cannot be digested by enzymes in the stomach and small intestine (Becker and Yu 2013), and many plant macronutrients also resist digestion, especially those in legumes and tubers, unless they are cooked (Zink and Lieberman 2016). Other food-processing techniques, such as fermentation, soaking, and sprouting, are also important for detoxifying starchy plants or removing antinutrients (Capparelli et al. 2011). Food processing may, however, also negatively affect enzymatic digestibility, solubility, and intestinal absorption of certain plant proteins. Proteins may become further polymerized during cooking (Yu et al. 2017), and high heat causes Maillard reactions between sugar and proteins that lead to irreversible protein modifications and a diverse range of indigestible compounds (Hemmler et al. 2018). Although it is well established that cooking modifies the protein, carbohydrate, and lipid contents of foods—and, consequently, bulk stable isotope ratios to some extent (Royer et al. 2017)—more work is required to establish how cooking affects amino acid stable isotope ratios.

Digestion and metabolic processes. We tend to be isotopically heavier than the food we eat, because digestive and metabolic processes preferentially oxidize isotopically lighter molecules that leave our bodies as carbon dioxide and urea. The first step in this multistep processes is conversion of carbohydrates, fats, and proteins into smaller molecules that can be absorbed by the lining of the small intestines (figure 2). Some nonessential amino acids, such as glutamine, glutamic acid, and aspartic acid, are catabolized extensively for oxidative fuel in the mucous membrane or used as building blocks for synthesizing other nonessential amino acids (Burrin and Stoll 2009). A lack of these nonessential amino acids in the diet can lead to increased catabolism of particular essential amino acids, such as leucine, which means that they become unavailable for the formation of structural tissues. Likewise, nutrient deprivation may increase the oxidation of dietary amino acids in the gut (Neis et al. 2015). Therefore, nonessential amino acids can be viewed as functionally essential if the supply does not meet metabolic demand (Womack and Rose 1947, Horvath et al. 1996).

The nutrients that escape primary digestion in the small intestine become available as a substrate for the microbiota community in the large intestine, the colon (Olimphant and Allen-Vercoe 2019). The primary fermentation products in the colon include various short-chain fatty acids and amine by-products from basic amino acids and phenols or indoles from aromatic amino acids (figure 2). The most important microbial fermentation by-products, the short-chain fatty acids, can be an intermediate for nonessential amino acid synthesis (Olimphant and Allen-Vercoe 2019). There is also evidence that a process is known as urea nitrogen salvaging may be responsible for gut microbial contributions of essential
amino acids, such as lysine and threonine, to the mammalian host (Metges 2000, Torrallardona et al. 2003). Urea, a highly water-soluble molecule, is the end nitrogenous product of protein catabolism. Once urea is passed via the blood into the gastrointestinal tract, microbes in both the small and large intestines may catabolize it into ammonia or use it as a nitrogen source for amino acid de novo synthesis (Stewart and Smith 2005). This process allows the host to salvage nitrogen, mostly in the form of ammonia and, to a much lesser degree, microbiologically synthesized amino acids. Therefore, a small part of the organism’s metabolic demand for essential amino acids may be met by the gut microbes but, as was reviewed by Fuller (2012), the degree to which dietary levels of proteins and complex carbohydrates, residence time of the digesta, and taxonomic assemblage of gut microbes affect essential amino acid supplementation to the host is not well understood (but see a recent rodent study by Newsome et al. 2020).

Digestive and metabolic processes alter amino acid stable isotope ratios. The most commonly used trophic amino acids for estimating trophic position, the δ¹⁵N of glutamic acid and proline, typically increase by approximately 5‰–8‰ per trophic step (McMahon and McCarthy 2016). δ¹⁵Nproline values are expected to mirror those of δ¹⁵Nglutamic acid because proline can be converted to glutamic acid in two catabolic steps that do not involve transamination (box 1; Fichman et al. 2015). In contrast to the trophic amino acids, the source amino acids cannot readily exchange nitrogen with the metabolic pool (box 1). As a result, commonly used source amino acids, such as phenylalanine and lysine, remain relatively unaltered as they move through the food chain (McMahon and McCarthy 2016). The δ¹⁵N change in source relative to trophic amino acids during trophic transfer is called the trophic discrimination factor (TDF). In hominin studies, the most applied TDF value for glutamic acid to phenylalanine (Glx–Phe) spacing is 7.5‰, although the degree to which this value is affected by turnover rates, nutritional demands and the macromolecular composition of the diets remains elusive.
A feeding trial with mice showed that higher protein led to smaller TDF\textsubscript{Glx–Phe} probably because proteins were used as an energy source instead of lipids (Whiteman et al. 2021). However, the TDF for proline to leucine spacing (TDF\textsubscript{Pro–Lys}) did not change significantly, indicating that these two amino acids may provide a more precise estimate of trophic position when the diet quality is unknown (figure 3). Threonine, which becomes $^{15}\text{N}$ depleted during trophic transfer, has been proposed as a new biomarker for protein consumption (Fuller and Petzke 2017), but more studies are needed to understand how $\delta^{15}\text{N}$\textsubscript{threonine} is affected by diet quality (Whiteman et al. 2021).

Interpreting dietary information from nonessential amino acid $\delta^{13}\text{C}$ patterns is complex because of the diverse pathways for producing intermediates used for nonessential amino acid \textit{de novo} synthesis (box 1). Furthermore, in addition to the three main exogenous sources of metabolic intermediates—carbohydrates, fats, and proteins—normal mammalian metabolism also involves the extensive turnover and degradation of endogenous amino acids and lipids. However, controlled feeding trials and epidemiological studies have shown the $\delta^{13}\text{C}$ of alanine, glycine, and glutamic acid to be a promising marker of the balance and content of dietary carbohydrates and fat (Choy et al. 2013, Wang et al. 2018, 2019, Yun et al. 2020, Johnson et al. 2021). It is possible to distinguish between these two nutrient fractions because the lipid moieties, glycerol and fatty acids, are $^{13}\text{C}$ depleted relative to proteins and carbohydrates owing to isotopic fractionation caused by the enzyme pyruvate dehydrogenase that connects the glycolysis and gluconeogenesis pathways with the tricarboxylic acid cycle (Deniro and Epstein 1977, Melzer and Schmidt 1987, Weber et al. 1997).

Biological variation also poses some challenges for $\delta^{15}\text{N}$\textsubscript{amino acid} and $\delta^{15}\text{N}$\textsubscript{amino acid} interpretation. For example, metabolic variations among species, individuals of the same species and even tissues of the same individual have been shown to affect $\delta^{15}\text{N}$\textsubscript{amino acid} discrimination (McMahon and McCarthy 2016). For $\delta^{15}\text{C}$\textsubscript{amino acid} a study with pigs fed varying proportions of terrestrial and marine proteins showed that valine, an essential amino acid, tended to be $^{13}\text{C}$ depleted in bone collagen and $^{13}\text{C}$ enriched in muscle tissue relative to diets (Webb et al. 2017). This finding is significant and reinforces the need to define tissue-specific $\delta^{15}\text{C}$\textsubscript{amino acid} offsets to reconstruct consumer diets and resource use accurately. Of particular importance to elucidating the degree of dietary, nutritional, and metabolic resolution available from $\delta^{15}\text{C}$\textsubscript{amino acid} and $\delta^{15}\text{N}$\textsubscript{amino acid} analyses of archeological and ecological tissues is the design of feeding trial studies that consist of naturally available foods rather than processed foods that bear little comparison to real-world scenarios.

**Isotopic variation of dietary sources.** The protein source of consumers will significantly influence the accuracy of estimates of the trophic position of amino acid stable isotope ratios. Based on $\delta^{15}\text{N}$\textsubscript{amino acid} analysis of cultivated and wild primary producers, Chikaraishi and colleagues (2009, 2010) first identified that the difference between the trophic and source amino acids, also termed the beta (β) value, is much lower in terrestrial than aquatic primary producers. However, it is becoming increasingly evident that it is not habitat type (terrestrial versus aquatic), but the degree of vascularization (formation of lignin rich structural tissues) that determines the β value in photoautotrophs (Boł et al. 2002, Styring et al. 2014, 2015, Kendall et al. 2019, Takizawa and Chikaraishi 2017, Takizawa et al. 2017). A recent meta-analysis showed the $\beta_{\text{Glx–Phe}}$ value to be $-6.6\%$ (standard deviation [SD] = 3.4$\%$) for vascular autotrophs and $3.3\%$ (SD = 1.8$\%$) for nonvascular autotrophs (Ramirez et al. 2021). The same study also showed that the β values of glutamic acid to leucine (2.5$\%$, SD = 1.6$\%$) are considerably less variable than the $\beta_{\text{Glx–Phe}}$ values in vascular plants. Since vascularization greatly affects trophic position estimates, it is important for paleodietary and paleoecological reconstructions to rely on additional archeological and biogeochemical evidence to define the most realistic β values. The following expression can be used to estimate trophic position, provided that both TDF and β values are well defined:

$$TP_{\text{CSIA}} = 1 + \frac{\delta^{15}\text{N}_{\text{amino acid}} - \delta^{15}\text{N}_{\text{amino acid}} - \beta}{\text{TDF}_{\text{amino acid}} - \text{S amino acid}}$$
where \( T \) and \( S \) signify trophic and source amino acids, respectively. In terms of carbon, essential amino acids are powerful tracers of basal resources in part because \( \delta^{13}C_{\text{essential amino acid}} \) values in most animals match those in source protein with little or no isotopic offsets, in contrast to the nonessential amino acids where the offsets are typically much greater (McMahon et al. 2010, Barreto-Curiel et al. 2017, Webb et al. 2017, Liu et al. 2018, Wang et al. 2018, 2019, Takizawa et al. 2020, Xu et al. 2021). However, more controlled feeding studies on mammalian model species are needed to validate the extent to which \( \delta^{13}C_{\text{essential amino acid}} \) values persist through multiple trophic transfers (Webb et al. 2017). Another key feature of the essential amino acids is that the primary organisms synthesizing them—algae, bacteria, fungi, and terrestrial plants—have distinct \( \delta^{13}C_{\text{amino acid}} \) patterns in which the relative differences among amino acids are consistent, regardless of the actual baseline \( \delta^{13}C \) values (Scott et al. 2006, Larsen et al. 2009, Larsen et al. 2013). These patterns are termed fingerprints when they are unique and unequivocal for a given basal resource. These diagnostic \( \delta^{13}C_{\text{essential amino acid}} \) fingerprints are well suited for retrospective analyses because they remain largely invariant across biogeochemical conditions and trophic transfer (Larsen et al. 2013, 2015, Lynch et al. 2016; Elliott Smith et al. 2018). For example, the range in \( \delta^{13}C \) values of algae grown under varying biogeochemical conditions can be as large as 12‰, but mean centering the \( \delta^{13}C_{\text{amino acid}} \) data can reduce the \( \delta^{13}C \) variability among individual essential amino acids by a factor of 10, which makes the fingerprinting approach a much more robust source tracer than bulk stable isotope ratios across time and space (Larsen et al. 2013, 2015). Mean centering is a technique to factor out baseline variability by subtracting the \( \delta^{13}C \) mean of all the essential amino acids from the \( \delta^{13}C \) of each of the individual essential amino acids. To accurately predict dietary sources with the fingerprinting approach, it is essential to obtain \( \delta^{13}C_{\text{amino acid}} \) data for the relevant food sources. The general term for the data used to create the classification model is training data. Examples of where more training data are needed for pinpointing particular hominin food sources in different contexts include plant organs, such as seeds, nuts, tubers and roots, and plants grown under different abiotic conditions (Paolini et al. 2015, Larsen et al. 2016, Jarman et al. 2017, Bontempo et al. 2020). Likewise, there are too little data available on secondary animal products, such as dairy, and the effects of food preparation on \( \delta^{13}C_{\text{amino acid}} \) patterns.

**Statistical analyses**

Given that amino acid stable isotope ratios involves \( \delta^{13}C \) and \( \delta^{15}N \) of a variety of amino acids, the resulting number of data points is of a magnitude order higher than those obtained from bulk stable isotope ratio analyses. For this reason, it is important to apply linear transformation techniques to identify the most important features in multivariate amino acid stable isotope ratio data sets, as is the case with the \( \delta^{13}C_{\text{amino acid}} \) fingerprinting approach mentioned above. Principal component analysis (PCA) is often the choice for exploring \( \delta^{13}C_{\text{amino acid}} \) variability and patterns in a data set because it is an unsupervised technique (i.e., the data are not grouped a priori) that seeks to maximize variability among samples while reducing the number of dimensions. Linear discriminant function analysis (LDA) is a supervised technique that seeks to maximize variability among the predefined groups or classes with the goal of predicting specific protein sources. The number of samples in each linear discriminant group should supersede the number of \( \delta^{13}C_{\text{amino acid}} \) variables, and the number of samples representing a food source should be greater than the number of essential amino acids. Overall, many amino acid variables as possible are desired to maximize dietary information. An important distinction between PCA and LDA is that only the latter technique assesses the variability of each \( \delta^{13}C_{\text{amino acid}} \) variable relative to the group mean. To achieve the same for PCA, it is necessary to factor out \( \delta^{13}C \) baseline variability by mean centering the data.

A multivariate approach based on paired \( \delta^{13}C_{\text{amino acid}} \) offsets can also differentiate consumers and predict major protein sources, but it has less predictive power than PCA and LDA, because it relies on three to four amino acids only. Archeologists have traditionally used paired amino acid offsets in conjunction with \( \delta^{13}C \) bulk or \( \delta^{13}C_{\text{amino acid}} \) baselines to identify consumption of high freshwater, marine, terrestrial C3, and terrestrial C4 protein sources. The offset between glycine and phenylalanine has been used to separate the high terrestrial C3 and high freshwater protein groups from the high terrestrial C4 and high marine protein groups, and the offset between valine and phenylalanine has been used to separate aquatic and terrestrial resources (Corr et al. 2005, Honch et al. 2012). In principle, it is possible to integrate paired \( \delta^{13}C_{\text{amino acid}} \) offsets with \( \delta^{13}C_{\text{amino acid}}, \delta^{14}N_{\text{amino acid}} \) and even bulk stable isotope ratio values in multivariate models, but only if these variables are completely independent of one another. For example, the amino acids used in paired \( \delta^{13}C_{\text{amino acid}} \) offsets must be omitted as single amino acid stable isotope ratio variables, and combining both bulk stable isotope ratios and amino acid stable isotope ratio variables for the same element is likewise problematic.

Interpretations of \( \delta^{13}C_{\text{amino acid}} \) and \( \delta^{15}N_{\text{amino acid}} \) data in ecological and archeological contexts can also be improved through combination with other isotopic proxies, such as \( \delta^{13}C_{\text{collagen}} \) or \( \delta^{14}C_{\text{collagen}} \) in Bayesian mixing models (Fernandes et al. 2014). In the context of paleoanthropology and archeology, the additional application of magnesium and zinc isotopes to trophic level exploration is also opening further proxy possibilities in this regard (Martin et al. 2015, Jaouen et al. 2016). The inclusion of other isotopic proxies must include the biosynthetic pathways associated with each proxy, however see (Fernandes et al. 2015). For instance, bone collagen nitrogen is sourced almost exclusively from dietary protein, whereas collagen carbon is sourced from a mixture of dietary protein, carbohydrate, and fat (Fernandes et al. 2012). It is also important to consider the research question being addressed with a mixing model. This is...
Analytical considerations

Researchers use either gas chromatography–combustion–isotope ratio mass spectrometry (GC–C–IRMS) or liquid chromatography–combustion–isotope ratio mass spectrometry (LC–C–IRMS) to determine amino acid stable isotope ratios. These methods each come with analytical advantages and drawbacks. Of the two systems, LC–C–IRMS produces the most reliable data, but it is only suited for carbon isotope analysis, the required sample amounts are very high, and some essential amino acid peaks cannot be baseline separated (Dunn et al. 2011, Smith et al. 2009). GC–C–IRMS can be used to determine both δ¹⁵N and δ¹³C values and the run times are much faster than with the LC–C–IRMS, meaning that it is increasingly the methodology of choice for δ¹³C amino acid and δ¹⁵N amino acid in archeological and anthropological contexts. However, the pre- and postanalytical steps are more cumbersome and complex.

The most demanding preanalytical step for preparing samples for GC–C–IRMS analysis is amino acid derivatization. This conversion of polar or nonvolatile compounds into relatively nonpolar or volatile products is usually done by esterifying the carboxylic acid group with an acified alcohol and acylating the amine, hydroxyl, and thiol groups (Corr et al. 2007b). Derivatization induces kinetic isotope effects and adds additional carbon atoms to the amino acids, and the only way to factor out these effects is by derivatizing a mixture of amino acids with known δ¹³C values (Docherty et al. 2001). To minimize the error propagations, it is important to use a surplus of reagents, select reagents and amino acid references with δ¹³C values approximating those of the samples of interest, and use a derivatization method that adds as few carbon atoms as possible (e.g., use short-chain alcohols for esterification; Corr et al. 2007a). Although derivatization does not add exogenous nitrogen, there may still be a kinetic isotope effect because of differing rates of chemical reaction of isotopically heavy and light atoms within a given molecule (i.e., isotopologues). This effect can be assessed by comparing the δ¹⁵N values of amino acid standards before and after derivatization (Whiteman et al. 2018).

The accuracy and precision of the isotopic analyses depend on the quality of gas chromatography separation, interface design, and isotopic calibration, which has been discussed in several papers and books (van Leeuwen et al. 2014, Jochmann and Schmidt 2015, Meier-Augenstein 2018). During runs, it is crucial to monitor the isotopic drift of analytical standards and reference materials, such as modern bones, and to ensure that scale normalization for the samples being measured is based on two or more reference analytes (Paul et al. 2007). A GC–C–IRMS system can be kept in good operating condition by monitoring for leakages, inspecting the combustion reactors, regularly changing inlet liners, and shortening or replacing gas chromatography columns. Similarly, it is important to check the relevant interfaces on LC–C–IRMS systems.

The most important proteinaceous tissues in an archeological or anthropological context are skin, hair, nails, ligaments, bones, and teeth. In the archeological record, researchers must often focus on the best-preserved tissue fragments for destructive isotope analysis. Depending on the age of the sample, these fragments can provide insights into the diet and nutrition of a particular life stage. Tissues such as bones, ligaments, and skin are remodeled throughout life but at different turnover rates according to the element in question and the age, sex, and physiological and pathological conditions of the individual (Hadjidakis and Androulakis 2007). In terms of bones, because ribs are remodeled at a much faster rate than femurs, the former will represent a more recent dietary history prior to death than the latter (Tieszen et al. 1983, Fahy et al. 2017). In contrast, tooth dentin and keratin excrences, such as hair and nails, are not remodeled after formation. Dentin is therefore useful for exploring diets during childhood and adolescence (Sandberg et al. 2014), and hair and nails can inform on diets in the months before death (O’Connell et al. 2001).

With regards to isotopic fractionation, feeding trials with pigs have shown that diet to collagen δ¹³C essential amino acid offsets (Δtissue–diet) can fall outside the 1% analytical uncertainty range. Notably, Δtissue–diet for valine ranged between 1.3‰ and −2.1‰ (Edgar Hare et al. 1991, Webb et al. 2017). We rule out the possibility that memory effects caused the higher than expected Δtissue–diet values because the experimental diets were fed to successive generations of pigs. Although isotopic discrimination during digestion and metabolic routing or microbial supplementation of essential amino acids may affect Δtissue–diet values, the most parsimonious explanation is probably sample treatment biases. For example, acid hydrolysis of collagen proteins can, in some cases, lead to Δtissue–diet values of 2‰–3‰ if the amino acid recovery rates are low (Jim et al. 2003). It is also worth noting from other animal feeding trials that Δtissue–diet values for the essential amino acids usually center around 0‰, and the corresponding values for the nonessential amino acids are much greater (McMahon et al. 2010, Barreto-Curiel et al. 2017, Webb et al. 2017, Wang et al. 2019).

Whether archeological tissues faithfully record diet and nutrition through amino acid stable isotope ratios will also depend on diagenetic biases. In terms of preservation, bone
Overview Articles

in cool environments where there was a relatively rapid burial, such as in the Denisova Cave in the Siberian Altai, where the oldest hominin collagen containing bone has been estimated to date to 195,000 years ago (Douka et al. 2019). For bones, collagen yields (more than 1%) and quality indicators such as atomic carbon to nitrogen ratios (2.9–3.5) and percentage nitrogen content (more than 0.5%) are the most important preservation criteria (Ambrose 1990, Brock et al. 2012). More recently, paleoproteomics has also been showing great promise for evaluating protein quality, because this technique can more accurately evaluate whether poorly preserved samples are suited for amino acid stable isotope ratio analysis than carbon to nitrogen ratio and other preservation quality indicators (Cleland et al. 2021).

It is well documented that the selection of pretreatment methodology can affect the final δ¹³C and δ¹⁵N values of measured bone collagen (Pestle et al. 2014) and food sources structures are sensitive to environmental fluctuations, such as humidity and temperature shifts, because they accelerate amino acid degradation by creating micro fissures and porous structures in biomineralized tissues (Grupe 1995, Maurer et al. 2014). Degradation and residues from conservation treatments can also introduce exogenous materials to tissues, but this is less of an issue for amino acid stable isotope ratios than for bulk stable isotope ratios because it is possible to extract and isolate amino acids bound in tissue proteins. According to a wool degradation study, soil microbial degradation of keratin appears not to be protein selective, which means that δ¹³C amino acid and δ¹⁵N amino acid values do not change significantly with degradation (von Holstein et al. 2014). Besides microbial degradation, the protein concentrations decrease with weathering and fossilization (Rapp Py-Daniel 2014). Therefore, the oldest bones suitable for stable isotope ratios of collagen are often found in cool environments where there was a relatively rapid burial, such as in the Denisova Cave in the Siberian Altai, where the oldest hominin collagen containing bone has been estimated to date to 195,000 years ago (Douka et al. 2019). For bones, collagen yields (more than 1%) and quality indicators such as atomic carbon to nitrogen ratios (2.9–3.5) and percentage nitrogen content (more than 0.5%) are the most important preservation criteria (Ambrose 1990, Brock et al. 2012). More recently, paleoproteomics has also been showing great promise for evaluating protein quality, because this technique can more accurately evaluate whether poorly preserved samples are suited for amino acid stable isotope ratio analysis than carbon to nitrogen ratio and other preservation quality indicators (Cleland et al. 2021).

It is well documented that the selection of pretreatment methodology can affect the final δ¹³C and δ¹⁵N values of measured bone collagen (Pestle et al. 2014) and food sources

Box 2. Schematic illustration of three different mixing models using δ¹³C values of essential amino acids as dietary tracers of fish, maize, and leafy vegetables and nuts.

The inner circles in figure 4 represent the weight or caloric values of essential amino acids and proteins relative to the total weight or calories of each food, and the outer circles the relative contribution of each food source. In this example for caloric estimates, the proportion of calories derived from proteins in each food source is 80% for fish, 13% for maize, and 40% for nuts and vegetables, and the relative caloric contribution of each food source is 28% from fish, 49% from maize, and 23% from nuts and vegetables. For simplicity, the essential amino acid proportions relative to proteins are assumed in the present example to be similar in each food source. The concentration independent model quantifies the relative essential amino acid contribution from each food source and carries the assumption that the relative essential amino acid proportions are similar in each food source. The protein concentration dependent model estimates the relative protein contribution from each food source by considering the essential amino acid relative to protein-only calories within each food source. The total macronutrient concentration dependent model estimates the relative caloric contribution of each food source by considering essential amino acid calories relative to total calories. It is important to note that the assimilation efficiency of macronutrients will not necessarily be 100% and may depend on other factors, such as cooking, digestibility, and antinutrients. Thus, effective concentration values that account for such aspects have to be employed in modeling. Some Bayesian software are capable of providing these separate estimates simultaneously when concentration values for essential amino acids and proteins are provided (Fernandes et al. 2012).

Figure 4. Schematic illustration of three different mixing models using δ¹³C values of essential amino acids as dietary tracers of fish, maize, and leafy vegetables and nuts.
Applications in archaeology and paleoecology

It should be clear from the above that, with robust analytical protocols in place, δ¹⁵Namine acid and δ¹³Camine acid measurement of hominin tissues has great potential for studying dietary adaptations and resource sourcing of one of the most prevalent terrestrial omnivores on the planet, humans (as well as their hominin ancestors). Although bulk bone collagen δ¹³C and δ¹⁵N analyses have been a staple of paleodietary investigations for four decades (Van Der Merwe and Vogel 1978, Krueger and Sullivan 1984, DeNiro 1985, Walker and Deniro 1986), they can have limited interpretive power because of a high ratio of food sources relative to isotope tracers and confounding bulk isotope values among resource groups (e.g., C₄ and marine resources; Fry 2013). By comparison, δ¹³Camino acid fingerprints have a much higher source specificity and, similar to the δ¹³Camine acid offsets used for inferring trophic position, they remain comparatively invariant across different environments (Larsen et al. 2013, 2015, Lynch et al. 2016, Elliott Smith et al. 2018). Therefore, δ¹³Camine acid and δ¹⁵Namine acid signals of ecological and archeological tissues are providing a clear improvement in the way we biochemically approach the study of past diets and ecosystems. Analyses of these tissues also play a role in improving the inferences that can be drawn from amino acid stable isotope ratio data in other disciplines. Under the compliance of strict ethical guidelines, the large number of human remains from known contexts that have been made available for studies with bulk stable isotope ratios or other biochemical approaches constitute a wealth of potential amino acid stable isotope ratio data that, in terms of quality, resolution, and distribution across space and time, is rarely attainable for other species.

The ability of δ¹³Namine acid values to overcome the issue of variable δ¹⁵N baselines is best illustrated through studies of nonomnivores fauna. To that end, we compiled bulk and δ¹³Namine acid data of modern frugivorous, insectivorous, and sanguinivorous bat tissues (Campbell et al. 2017). Please find the statistical methods for this article in supplemental appendix S1. Despite the different trophic positions of the bats, there is a lack of significant differences in bulk δ¹⁵N values among the three groups due to environmental variations (figure 5a). By contrast, trophic position estimates based on δ¹³Ngly–Phe values clearly reveal that the three groups are different and accord with observations of their foraging ecology (figure 5c). In a different case study, bulk δ¹⁵N values of Pleistocene horse bone collagen in Western Europe and Yukon are suggestive of a trophic distinction between horses inhabiting these regions (figure 5b; Schwartz-Narbonne et al. 2015). However, δ¹³Ngly–Phe analysis of the same samples demonstrates this to be a product of baseline δ¹⁵N variation, with equids actually, and, unsurprisingly, inhabiting the same trophic position, in both parts of the world (figure 5d).

Despite the more complex nature of inferring trophic position from δ¹³Ngly–Phe in omnivores, this proxy has been used successfully to provide detailed dietary information of humans and their hominin relatives. For example, Richards and Trinkaus (2009) concluded, on the basis of both archeological and bulk isotopic data, that Neanderthal diets were heavily meat based, without detectable fish or plant inputs. However, bulk δ¹⁵N methods lack source specificity and may be influenced by regional environmental δ¹⁵N variations (Wang et al. 2014, Styring et al. 2016). Recent baseline-independent δ¹³Ngly–Phe analyses of bone collagen from Neanderthals and associated carnivores (e.g., hyaenas and wolves) and herbivores (e.g., mammoths, equids, and deer) have largely confirmed Neanderthals as high trophic level carnivores at sites such as Les Cottés and the Grotte du Renne, in France. However, they have also highlighted dietary contributions of plants in locations such as Spy Cave in Belgium (Naito et al. 2016b, Jaouen et al. 2019). Despite this improved resolution, we still know little about the dietary versatility of Neanderthals, their ability to adapt their foraging strategies to local conditions, and whether their resource gathering strategies were distinct from our own species. Systematic δ¹³Ngly–Phe work comparing Neanderthals and Homo sapiens in different regions, as well as analysis of Neanderthals across their ever
expanding known geographical range, promises to provide further important insights into the dietary adaptability of this hominin group in the future (Belmaker and Hovers 2011, Hallin et al. 2012, Henry et al. 2017).

Although δ15Namino acids is emerging as an indispensable method for dietary reconstruction, it has limited ability to independently determine proportion contributions of terrestrial versus aquatic resources (i.e., the correct β values), which, in turn, can lead to erroneous estimations of trophic positions (see figure 5). This limitation can be reduced in hominin dietary studies by measuring the local δ15N values of freshwater fish, terrestrial plants, terrestrial meat, and seafood (Naito et al. 2016a, Drucker et al. 2017, Itahashi et al. 2019). For example, in a δ15Namino acid study of bone remains from hunter–gatherer and Neolithic populations in the upper Tigris region in the Near East, Itahashi and colleagues (2019) used faunal remains to show that δ15Nphenylalanine values of freshwater fish are lower than those of terrestrial herbivores and carnivores. By employing a trophic position model assuming terrestrial protein contributions, Itahashi and colleagues (2019) inferred high aquatic protein consumption when human bone collagen had a combination of low δ15Nphenylalanine and high trophic position values (Itahashi et al. 2019). Although such approaches rest on a number of assumptions, in relation to both the bulk δ13C and δ15N values of faunal remains and the relative abundance of faunal taxa in each excavation layer, the study demonstrated how δ15Namino acid can help to shed light on resource use—in this case, how a shift from hunting and gathering to farming decreased human dependence on aquatic resources.

The issue of estimating the β values of hominins living off of mixed terrestrial and aquatic diets can also be addressed by using δ13Cessential amino acid fingerprints, as exemplified by a joint δ15Namino acid and δ13Cessential amino acid data set from prehistoric communities on Rapa Nui (Easter Island) and their potential food sources represented by a mixture of archeological and modern rat, chicken, plant food, and different marine resources (Jarman et al. 2017). The δ13Cessential amino acid fingerprints and the accompanying concentration-independent mixing model (representing dietary essential amino acids) estimated that the islanders relied much more on marine dietary resources than was previously thought, highlighting the finely tuned environmental adaptations and social resilience of humans on an island previously simplistically discussed as a classic case study of human overexploitation and societal collapse (Diamond 2005). In terms of estimating the islanders’ trophic position, Jarman and colleagues (2017) compared three different dietary scenarios: β values based on fully marine, half marine half terrestrial diets, and fully terrestrial diets. Only the 50–50 scenario produced somewhat realistic trophic positions, albeit a quarter of the individuals had trophic positions below 2. This low range can be attributed to an overestimation of marine resources. Therefore, to generate realistic β values, we use the estimates produced by Commendador and colleagues (2019) for marine and terrestrial caloric contributions with a concentration-dependent (representing dietary carbon) rather than with a concentration-independent mixing model (figure 6; Jarman et al. 2017). Despite some uncertainty in the proportions of marine to terrestrial contributions, the revised β value results in more realistic trophic position estimates ranging between 2.1 and 2.6 (figure 6).

Acknowledging the nuances in routing and metabolic processes and how different δ13Cessential amino acid and δ15Namino acid proxies can track these processes, amino acid stable isotope ratios analyses offer opportunities for archeological and paleoecological researchers despite the difficulties presented above. There are remarkably few studies that combine the power of δ13Cessential amino acid and δ15Namino acid analyses on the same samples to simultaneously reveal more high-resolution insights into...
diet, environmental impacts, and trophic relationships. Four recent studies from widely different archeological settings—Neanderthals in France (Naito et al. 2016a, Jaouen et al. 2019), prehistoric humans on Rapa Nui (Jarman et al. 2017), and Romans from Herculaneum—combined δ¹³C essential amino acid and δ¹⁵N amino acid analyses to obtain more robust evidence of dietary patterns (Soncin et al. 2021). The Neanderthal studies applied δ¹³C essential amino acid in conjunction with zooarchaeological evidence to rule out the possibility that food processing and consumption of fish and young mammals contributed to unusually elevated collagen δ¹⁵N values, thereby supporting that Neanderthals were indeed top-level carnivores. The Rapa Nui study combined δ¹³C amino acid and δ¹⁵N amino acid data to show that islanders were more versatile in their foraging strategies than previously assumed. Finally, the Herculaneum study relied on abundant food remains to make a direct comparison between human bone collagen and food isotopic values for the essential amino acids and source-trophic amino acids to increase the precision of Bayesian dietary estimates.

A cornerstone for inferring past human diets is analyzing food sources that accurately represent past hominin diets. As was mentioned earlier, the breadth of archeologically relevant training data is still limited. Another constraint is the relatively low number of essential amino acid variables reported in archeological studies to date. Despite these limitations, much can be gleaned from already published δ¹³C amino acid data (Ma et al. 2021). To illustrate this, we compiled δ¹³C essential amino acid data (leucine, lysine, phenylalanine, and valine) from archeological studies using fauna as proxies for marine, terrestrial C₃, and terrestrial C₄ protein sources, and two human populations from C₃– and C₄-dominated biomes, respectively. One population was from Herculaneum (Rome, 79 CE) and is documented as living on a diet composed primarily of animal–plant terrestrial C₃ foods and, to a lesser extent, marine fish (Soncin et al. 2021). The other population from Nancheng (China, 2000–1600 BCE) was argued to be reliant on millet and fauna that foraged on mixed C₃ or C₄ vegetation (Ma et al. 2021). The LDA based prediction of human diets largely accord with expectations (figure 7). Most of the Herculaneum individuals cluster with the herbivorous C₃ fauna indicating that most of their proteins derived from terrestrial sources. The remaining Herculaneum individuals, with one exception (marked with an asterisk), are skewed toward marine protein sources, which may reflect that fish protein contribution is much greater than overall fish caloric contributions (estimated to approximately 10% by Soncin et al. 2021). This is plausible given that proteins in marine fishes represent approximately 80% of their total calories, against 12% in ancient cereals (Liaset and Espe 2008, Boukid et al. 2018). However, we cannot exclude the possibility that tissue to diet isotopic offsets, Δ tissue-diet, shifted LD scores toward marine sources. All the Nancheng individuals except one (marked with an asterisk) plot with the herbivore C₃ faunal group, but with an offset along LD2 owing to the aforementioned Δ tissue-diet or the fact that nonfaunal protein sources, such as millet grains, have slightly different δ¹³C essential amino acid fingerprints than the C₄ grasses eaten by the herbivorous fauna. It is also noteworthy that the elongated ellipse of the C₄ fauna group along LD1 indicates that some specimens relied more on C₃ plants than others. The LDA cannot adequately explain the diets of the two outliers: It is unlikely that the Nancheng individual predominantly relied on marine proteins given the site’s distance from the ocean, and δ¹³C amino acid baseline values of the Herculaneum individual bins it with the other individuals from the site.

To further explore how data from human osteological remains can contribute to an increased interpretive power of different δ¹³C amino acid based dietary estimates, we compiled published δ¹³C amino acid data of humans from archeological sites applying the criteria that the data contained at least four essential amino acid variables (leucine, lysine, phenylalanine, and valine). In addition to the Herculaneum and Nancheng
individuals, we compiled data from four other sites: The first two sites are Jabuticabeira II, Piacaguera, and Galheta IV (6700 to 1000 calibrated years before the present), in Brazil (Colonese et al. 2014), where humans mainly subsisted on a mixture of aquatic resources and plant foods. The other two sites are the Nukdo shell midden (Korea, 550 BCE to 1 CE; Choy et al. 2010), where humans subsisted on mixed marine and terrestrial diets, and Pica 8 (Peru, 1050–500 calibrated years before the present), in Brazil (Colonese et al. 2014), where humans mainly subsisted on maize-dominated diet (Mora et al. 2018). Based on the PCA of the four available essential amino acids and six nonessential amino acids, we found that the six populations clustered separately but with the two Brazilian populations grouping adjacent (figure 8a). Both PC1 and PC2 are needed to separate consumers with marine- and terrestrial-dominated diets. The fact that several of the Nukdo individuals fall next to the two Brazilian groups is likely indicative of a high marine protein intake. The disparity in principal components scores and $\delta^{13}$C$_{amino}$ acid baselines between the Brazilian and the Herculaneum individuals (figure 8b) supports the fact that marine fish made a smaller dietary contribution in the latter case (Colonese et al. 2014, Soncin et al. 2021). In terms of phenylalanine versus valine separating terrestrial from aquatic consumers (Honch et al. 2012, Webb et al. 2017), our data suggest phenylalanine versus the three using essential amino acids (valine, leucine, and lysine) is a more powerful approach for separating these dietary groups.

The comparative analysis groups the Herculaneum outlier with other individuals from the same site suggesting that analytical uncertainty of an essential amino acid variable explained its outlier position in the LDA. This finding reinforces the importance of analyzing as many amino acid variables as possible when making robust dietary inferences. The comparative analysis also bins the Nancheng outlier with other individuals from the same site, but it remains more $^{13}$C depleted than the others. The most parsimonious explanation for this and the two $^{13}$C-depleted Galheta and Pica 8 individuals (marked with asterisks in figure 8a) is a contribution of C$_{3}$-derived proteins. In terms of lysine and phenylalanine separating the two C$_{3}$-dominated biomes, Nancheng and Pica 8, it may be relevant to investigate how protein quality and food preparation affect lysine to phenylalanine $\delta^{13}$C spacing. Lysine is the first limiting essential amino acid and phenylalanine the last limiting essential amino acid in both maize and pearl millet (Anitha et al. 2020, Wiedemair et al. 2020). For this reason, human $\delta^{13}$C$_{lysine}$ values are likely to be much more sensitive than $\delta^{13}$C$_{phenylalanine}$ values to protein supplementation from animal foods and food processing increasing digestibility. With regards to the nonessential amino acids, it is noteworthy that the eigenvectors of glycine and alanine point in opposite directions, confirming observations from human epidemiological studies and animal feeding trials that dietary macronutrients are routed differently to these two glycolytic amino acids (Wang et al. 2019, Yun et al. 2020). These and potentially many other observations pertaining to the relationship among amino acid variables highlight how comparative studies based on archeological humans with relatively robust dietary contexts can provide clues on how to interpret $\delta^{13}$C$_{amino}$ acid data.

**Perspectives for hominin studies**

Bulk $\delta^{13}$C and $\delta^{15}$N analysis of ecological and archeological proteinaceous tissues has long served as an important avenue for exploring the diets and life histories of hominins (Sehrawat and Kaur 2017). However, issues of equifinality, influences of environmental variation, and a general coarseness of interpretation have demanded new biomarkers for discerning variation in resource use, the consumption of different macronutrients, and interorganism relationships on local, regional, and even global scales. We hope to have demonstrated that $\delta^{13}$C$_{amino}$ acid and $\delta^{15}$N$_{amino}$ acid approaches have such a potential. Although applications remain limited in archeology, anthropology, and (paleo)ecology, the joint application of these compound-specific isotopic
methodologies offers the opportunity of pinpointing trends of dietary reliance, perhaps even to individual resources in some cases, and determining metabolic patterns and nutritional deficiencies. For these methods to reach their full potential and to be used in a proper and uniform manner, it is essential that a wider, global multidisciplinary audience is aware of both their promise and current limitations.

As with many novel methodological tracer approaches, the enthusiastic application of δ¹³C amino acid and δ¹⁵N amino acid approaches should be tempered by a need for consistency in preparation, measurement, and correction for added carbon during derivatization and for the use of reference standards and samples (Roberts et al. 2018, Meier-Augenstein and Schimmelmann 2019). Interlaboratory comparison and shared protocols are essential, because the potential for δ¹³C amino acid and δ¹⁵N amino acid variation as a result of derivatization and measurement methodologies are becoming increasingly apparent (Ohkouchi et al. 2017, Yarnes and Herszage 2017), and compound-specific practitioners should follow the work done on bulk isotopic analysis of proteinaceous materials in this regard (Brand et al. 2014). To facilitate interlaboratory comparisons of amino acid stable isotope ratio data, it would be particularly advantageous if, with every batch of archeological samples, the research community agreed to include a couple of globally available collagen samples extracted from modern faunal bones. Ideally, these samples should encompass the naturally occurring range of amino acid stable isotope ratio values (e.g., a C₃ herbivore and a marine carnivore, respectively). More open, thorough discussions on the topic of analytical issues are essential if growing δ¹³C amino acid and δ¹⁵N amino acid data sets in archeology are going to be comparable and useful for future meta-analyses and building understandings of how modern variation can be related to archeological problems.

Our multiple variate analysis of amino acid stable isotope ratio data demonstrates the importance of embracing as many amino acid variables as possible for robust dietary inferences and the need for expanding training and comparative data. It is crucial that researchers publish and perform quality control on as many amino acid variables as possible. In this way, larger reference data sets and multivariate evaluations of patterns can help to explore the full utility of δ¹³C amino acid and δ¹⁵N amino acid variability. With regards to δ¹³C, the nonessential amino acids are more complex to interpret than the essential amino acids, but they do open the door for the development of a series of additional biomarker approaches to diet reconstruction, such as inferring macromolecular composition (Larsen et al. 2022). In the case of δ¹⁵N, source-trophic pairs, such as proline and lysine, or glutamic acid and lycine, may provide more consistent trophic position estimates for hominins than the commonly used glutamic acid-phenylalanine pair. Moving beyond trophic position could potentially yield a more holistic understanding of the nutritional status of consumers, such as employing δ¹⁵Nbreanne as a biomarker for protein intake (Fuller and Petzke 2017). The aspiration to apply amino acid stable isotope ratio analyses for paleodiетary reconstruction should be juxtaposed against their relatively high analytical costs. However, ongoing advances in analytical approaches, an expansion of laboratories with the capacity to measure δ¹³C amino acid and δ¹⁵N amino acid, and emerging exposure in archeological and anthropological disciplines promise to see amino acid

Figure 8. Principal component analysis of compiled δ¹³C amino acid data from human archeological bone or tendon collagen. (a) The principal component scores based on mean-centered δ¹³C values of four essential amino acids (leucine, lysine, phenylalanine, valine) and six nonessential amino acids (alanine, aspartic acid, glutamic acid, glycine, hydroxyproline, proline). (b) A mirror of panel (a) but color coded according to their mean δ¹³C amino acid values. The median discriminant values of six sites based on the first three principal component (PC) scores (accounting for 75.5% of the variation) were significantly different (Pillai’s trace = 2.14, F(15,157) = 24.4, p < .001). The convex hulls represent the maximum range of the PC1 and PC2 scores of each group, and the arrows represent the relative weightings of the independent variables for creating the principal component analysis. The symbols marked with asterisks denote outlier individuals with deviating linear discriminant scores (see figure 7) or δ¹³C amino acid baselines. References: Jabuticabeira II, Piacaguera, and Galheta IV (Brazil, 6700 to 1000 calibrated years before the present; Colonese et al. 2014), Herculaneum (Rome, 79 CE; Soncin et al. 2021), Nancheng (China, 2000–1600 BCE; Ma et al. 2021), Nukdo shell midden (Korea, 550 BCE to 1 CE; Choy et al. 2010), and Pica 8 (Peru, 1050–500 calibrated years before the present; Mora et al. 2018). See supplemental appendix S2 for sample information. Image: Silhouettes from PhyloPic (http://phylopic.org) under a Creative Commons license.

https://academic.oup.com/bioscience
stable isotope ratios become an increasingly regular part of paleodiory and paleoecological research agendas.

Data from modern populations can increase our ability to associate particular amino acid stable isotope ratio patterns with biomes and regions, but dietary homogenization and the increasing globalization of the food supply is making such efforts increasingly difficult. For instance, the contrasts between modern and ancient gut microbiomes (Wibowo et al. 2021) remain uncertain with regards to amino acid stable isotope ratio patterns. Despite these caveats, modern amino acid stable isotope ratio studies will be crucial for developing taxon or biome specific fingerprints that can be used as baselines for the interpretation of human data in the past. Observational studies of humans and other primates will also be important for exploring how amino acid stable isotope ratio variability is related to physiological and dietary factors. Besides applying $\delta^{13}$Cmonospecific amino acid values as biomarkers of particular foodstuffs (Choy et al. 2013, Yun et al. 2020, Johnson et al. 2021), researchers are also investigating the extent $\delta^{13}$Camine acid Values can inform about biometric traits, such as body mass index, age, and sex (Jackson et al. 2015, Matos and Jackson 2020). More feeding experiments on pigs and other mammalian model species are needed to improve contextualization of omnivore-specific $\delta^{13}$Camine acid Patterns (Edgar Hare et al. 1991, Webb et al. 2017). This is particularly true for nutritional stressors, such as starvation and a lack or an excess of dietary proteins (Doi et al. 2017, Fry and Carter 2019). Moreover, a growing number of projects using modern human and primate hair and teeth, within appropriate ethically designed studies, have been used to better understand patterns of bulk isotope variation connected to the environment (Macho and Lee-Thorp 2014), season (Oelze 2016), weaning (Dailey-Curtis et al. 2017, Fry and Carter 2019), and physiological stress (D’Ortenzio et al. 2015, Crowley et al. 2016), providing useful models for future amino acid stable isotope ratio research. Much can be learned as well from human studies applying bulk stable isotope ratios as health indicators (Petzke et al. 2010, O’Brien 2015).

The adage that something is greater than the sum of its parts is particularly true for archeological amino acid stable isotope ratio data. The literacy of amino acid stable isotope ratio data can be enhanced by tying them to contextual information such as the environment humans lived in, social conditions and the artefacts they left behind, and then comparing this information across different populations. For example, as our compilation data set shows, determining the amino acid stable isotope ratios of humans living in different regions with different observed diets (based on archeobotany, archeozoology, and, in some cases, historical records) can provide a point of exploration as to how spacing of $\delta^{13}$C and $\delta^{15}$N among different amino acids relates to diet or ecology. This may, in turn, provide targeted areas for future research. Similarly, a combination of $\delta^{13}$Camine acid and $\delta^{15}$Namine acid from archeological contexts with DNA studies of the microbiome and other multidisciplinary approaches to food preparation may provide insights into how specific biological digestive processes and cultural manipulation of foodstuffs (e.g., fermentation) might influence $\delta^{13}$Camine acid and $\delta^{15}$Namine acid variability. As we demonstrated in the present article, amino acid stable isotope ratio analyses of hominin remains, where preservation conditions allow, hold a vast potential for advancing and nuancing our understanding of past human–environment interactions. A deep understanding of our past is important, because increasing social complexity and implementation of new technologies in human societies have not only shaped the diet of our species but have also altered the habitats and resource base of other species (Moll et al. 2021). To fulfill the promise of amino acid stable isotope ratios, both modern and archeological data sets will be essential for providing important reference points for multivariate modeling and probing of isotopic and dietary variation.

Acknowledgments

We would like to thank the Max Planck Society for funding this work.

Supplemental material

Supplemental data are available at BIOSCI online.

References cited

Aiello LC, Wheeler P. 1995. The expensive-tissue hypothesis: The brain and the digestive system in human and primate evolution. Current Anthropology 36: 199–221.

Ambrose SH. 1990. Preparation and characterization of bone and tooth collagen for isotopic analysis. Journal of Archaeological Science 17: 431–451.

Anitha S, Govindaraj M, Kane-Potaka J. 2020. Balanced amino acid and higher micronutrients in millets complements legumes for improved human dietary nutrition. Cereal Chemistry 97: 74–84.

Armelagos GJ. 2014. Brain evolution, the determinates of food choice, and the omnivore’s dilemma. Critical Reviews in Food Science and Nutrition 54: 1330–1341.

Attewell L, Kovarovic K, Kendall JR. 2015. Fire in the Plio–Pleistocene: The functions of hominin fire use, and the mechanistic, developmental and evolutionary consequences. Journal of Anthropological Sciences 93: 1–20.

Barreto-Curiel F, Focken U, D’Abramo LR, Viana MT. 2017. Metabolism of Seriola lalandi during starvation as revealed by fatty acid analysis and compound-specific analysis of stable isotopes within amino acids. PLOS ONE 12: e0170124.

Becker PM, Yu P. 2013. What makes protein indigestible from tissue-related, cellular, and molecular aspects? Molecular Nutrition and Food Research 57: 1695–1707.

Belmaker M, Hovers E. 2011. Ecological change and the extinction of the Levantine Neanderthals: Implications from a diachronic study of micromammals from Amud Cave, Israel. Quaternary Science Reviews 30: 3196–3209.

Beuning KRM, Zimmerman KA, Ivory SJ, Cohen AS. 2011. Vegetation functions of hominin fire use, and the mechanistic, developmental and evolutionary consequences. Journal of Archaeological Science 36: 3196–3209.

Boeving Beuning KRM, Zimmerman KA, Ivory SJ, Cohen AS. 2011. Vegetation response to glacial–interglacial climate variability near Lake Malawi in the southern African tropics. Palaeogeography, Palaeoclimatology, Palaeoecology 303: 81–92.

Bol R, Ostlie NJ, Petzke KJ. 2002. Compound specific plant amino acid $\delta^{15}$N values differ with functional plant strategies in temperate grassland. Journal of Plant Nutrition and Soil Science (Zeitschrift fur Pflanzennahrung und Bodenkunde) 165: 661–667.
Overview Articles

Bontempo L, van Leeuwen KA, Paulini M, Holst Laursen K, Micheloni C, Premier PD, Ryan D, Cumin F. 2020. Bulk and compound-specific stable isotope ratio analysis for authenticity testing of organically grown tomatoes. Food Chemistry 318: 126426.

Borman A, Wood TR, Black HC, Anderson EG, Osterling MJ, Womack M, Rose WC. 1946. The role of arginine in growth with some observations on the effects of arginineic acid. Journal of Biological Chemistry 166: 585–594.

Boukidi F, Folloni S, Sforza S, Vittadini E, Prandi B. 2018. Current trends in ancient grains-based foodstuffs: Insights into nutritional aspects and technological applications. Comprehensive Reviews in Food Science and Food Safety 17: 123–136.

Bowenis JM, Ascough PL, Cook GT, Murray I, Bonsall CJR. 2017. Using stable isotopes and a Bayesian mixing model (FRUITS) to investigate diet at the early Neolithic site of Carding Mill Bay, Scotland. Nature 59: 1275–1294.

Brand WA, Coplen TB, Vogl J, Rosner M, Prohaska T. 2014. Assessment of international reference materials for isotope-ratio analysis (IUPAC Technical Report). Pure and Applied Chemistry 86: 425–467.

Brock F, Wood R, Higham TFG, Ditchfield P, Bayliss A, Ramsey C. 2012. Reliability of nitrogen content (%N) and carbon: Nitrogen atomic ratios (CN) as indicators of collagen preservation suitable for radiocarbon dating, Radiocarbon 54: 879–886.

Burrin DG, Stoll B. 2009. Metabolic fate and function of dietary glutamate in the gut. American Journal of Clinical Nutrition 90: 8505–8565.

Campbell DJ, Nelson DM, Ogawa NO, Chikaraishi Y, Ohkouchi N. 2017. Trophic position and dietary breadth of bats revealed by nitrogen isotopic composition of amino acids. Scientific Reports 7: 15932.

Candela M, Astiasaran I, Bello J. 1997. Cooking and warm-holding: Effect on general composition and amino acids of kidney beans (Phaseolus vulgaris), chickpeas (Cicer arietinum), and lentils (Lens culinaris). Journal of Agricultural and Food Chemistry 45: 4763–4767.

Capparelli A, Valamoti SM, Wollstonecroft MM. 2011. After the harvest: Investigating the role of food processing in past human societies. Archaeological and Anthropological Sciences 3: 1–5.

Chau C-F, Cheung PC-K, Wong Y-S. 1997. Effects of cooking on content of amino acids and antinutrients in three Chinese indigenous legume seeds. Journal of the Science of Food and Agriculture 75: 447–452.

Cheung C, Szpak P. 2020. Interpreting past human diets using stable isotope mixing models. Journal of Archaeological Method and Theory 28: 1106–1142.

Chikaraishi Y, Ogawa NO, Kashiyama Y, Takano Y, Suga H, Tomitani A, Chikaraishi Y, Ogawa NO, Khokouchi N. 2009. Determination of aquatic food-web structure based on compound-specific nitrogen isotopic composition of amino acids. Limnology and Oceanography: Methods 7: 740–750.

Chikaraishi Y, Ogawa NO, Ohkouchi N. 2010. Further evaluation of the trophic level estimation based on nitrogen isotopic composition of amino acids. Pages 37–51 in Ohkouchi N, Tayasa I, Kobu K, Ohkouchi N, eds. Earth, Life, and Isotopes. Kyoto University Press.

Choy K, Nash SH, Kristal AR, Hopkins S, Boyer BB, O’Brien DM. 2013. The carbon isotope ratio of alanine in red blood cells is a new candidate biomarker of sugar-sweetened beverage intake. Journal of Nutrition 143: 878–884.

Choy K, Smith CI, Fuller BT, Richards MP. 2010. Investigation of amino acid δ13C signatures in bone collagen to reconstruct human palaeodiet using liquid chromatography–isotope ratio mass spectrometry. Geochimica et Cosmochimica Acta 74: 6093–6111.

Cleland TP, Sarancha JJ, France CAM. 2021. Proteomic profile of bone “collagen” extracted for stable isotopes: Implications for bulk and single amino acid analyses. Rapid Communications in Mass Spectrometry 35: e9025.

Colonese AC, et al. 2014. Long-term resilience of Late Holocene coastal subsistence system in southeastern South America. PLOS ONE 9: e93854.

Commodan AR, Finney BP, Fuller BT, Tromp M, Dudgeon JV. 2019. Multiproxy isotopic analyses of human skeletal material from Rapa Nui: Evaluating the evidence from carbonates, bulk collagen, and amino acids. American Journal of Physical Anthropology 169: 714–729.

Cornellio AM, de Bittencourt-Navarrete RE, de Bittencourt Brum R, Queiroz CM, Costa MR. 2016. Human brain expansion during evolution is independent of fire control and cooking. Frontiers in Neuroscience 10: 167.

Cott LR, Berstan R, Evershed RP. 2007a. Development of N-acetyl methyl ester derivatives for the determination of δ13C values of amino acids using gas chromatography–combustion–isotope ratio mass spectrometry. Analytical Chemistry 79: 9082–9090.

Cott LR, Berstan R, Evershed RP. 2007b. Optimisation of derivatisation procedures for the determination of δ13C values of amino acids by gas chromatography/combustion/isotope ratio mass spectrometry. Rapid Communications in Mass Spectrometry 21: 3759–3771.

Cott LR, Sealy JC, Horton MC, Evershed RP. 2005. A novel marine dietary indicator utilising compound-specific bone collagen amino acid δ13C values of ancient humans. Journal of Archaeological Science 32: 321–330.

Crowley BE, Reitsema LJ, Oelze VM, Sponeheimer M. 2016. Advances in primate stable isotope ecology: Achievements and future prospects. American Journal of Primatology 78: 995–1003.

Dailey-Chwallbó T, et al. 2020. Weaning and stunting affect nitrogen and carbon stable isotope natural abundances in the hair of young children. Scientific Reports 10: 2522.

DeNiro MJ. 1985. Postmortem preservation and alteration of in vivo bone collagen isotope ratios in relation to palaeodietary reconstruction. Nature 317: 806–809.

Deniro MJ, Epstein S. 1977. Mechanism of carbon isotope fractionation associated with lipid-synthesis. Science 197: 261–263.

Diamond J. 2005. Collapse: How Societies Choose to Fall or Succeed. Penguin.

 Docherty G, Jones V, Evershed RP. 2001. Practical and theoretical considerations in the gas chromatography/combustion/isotope ratio mass spectrometry δ13C analysis of small polyfunctional compounds. Rapid Communications in Mass Spectrometry 15: 730–738.

Doi H, Akamatsu F, González AL. 2017. Starvation effects on nitrogen and carbon stable isotopes of animals: An insight from meta-analysis of fasting experiments. Royal Society Open Science 4: 170633.

Domínguez-Rodrigo M, Bunz HT, Mabulla AZ, Baquadano E, Uribelarrea D, Pérez-González A, Gidna A, Yravedra J, Diez-Martín F, Egeland CP. 2014. On meat eating and human evolution: A taphonomic analysis of BK4b (Upper Bed II, Olдуvae Gorge, Tanzania), and its bearing on hominin megafaunal consumption. Quaternary International 322: 129–152.

D’Orentino L, Brickley M, Schwarz H, Prowse T. 2015. You are not what you eat during physiological stress: Isotopic evaluation of human hair. American Journal of Physical Anthropology 157: 374–388.

Douxka K, et al. 2019. Age estimates for hominin fossils and the onset of the Upper Palaeolithic at Denisova Cave. Nature 565: 640–644.

Drucker DG, et al. 2017. Isotopic analyses suggest mammoth and plant in the diet of the oldest anatomically modern humans from far southeast Europe. Scientific Reports 7: 6833.

Dunn PJH, Honch NV, Evershed RP. 2011. Comparison of liquid chromatography–isotope ratio mass spectrometry (LC/IRMS) and gas chromatography–combustion–isotope ratio mass spectrometry (GC/C/IRMS) for the determination of collagen amino acid δ13C values for palaeodietary and palaeoecological reconstruction. Rapid Communications in Mass Spectrometry 25: 3095–3101.

Edgar Hare P, Fogel MI, Stafford TW, Mitchell AD, Hoering TC. 1991. The isotopic composition of carbon and nitrogen in individual amino acids isolated from modern and fossil proteins. Journal of Archaeological Science 18: 277–292.

Elliott Smith EA, Harrod C, Newsome SD. 2018. The importance of kelp to an intertidal ecosystem varies by trophic level: Insights from amino acid δ13C analysis. Ecosphere 9: 1–14.

https://academic.oup.com/bioscience

July 2022 / Vol. 72 No. 7  •  BioScience 633
as a carbon source tracer in marine sediments: Effects of algal growth conditions and sedimentary diageneis. Biogeochemistry 12: 4979–4992.
Larsen T, Polyacter MM, Holmstrup M, D’Annibale A, Maraldo K, Andersen N, Eriksen J. 2016. Substantial nutritional contribution of bacterial amino acids to earthworms and enchytraeids: A case study from organic grasslands. Soil Biology and Biochemistry 99: 21–27.
Larsen T, Yokoyama Y, Fernandes R. 2018. Radiocarbon in ecology: Insights and perspectives from aquatic and terrestrial studies. Methods in Ecology and Evolution 9: 181–190.
Larsen T, Wang YV, Wan AHL. 2022. Tracing the trophic fate of aquafeed macronutrients with carbon and amino isotope ratios of amino acids. Frontiers in Marine Science 9: 813961.
Lee-Thorp JA, Sealy JC, Van Der Merwe NJ. 1989. Stable carbon isotope ratio differences between bone collagen and bone apatite, and their relationship to diet. Journal of Archaeological Science 16: 585–599.
Liaset B, Espe M. 2008. Nutritional composition of soluble and insoluble fractions obtained by enzymatic hydrolysis of fish–raw materials. Process Biochemistry 43: 42–48.
Liu H-Z, Luo L, Cai D-L. 2018. Stable carbon isotopic analysis of amino acids in a simplified food chain consisting of the green alga Chlorella spp., the calanoid copepod Calanassimius, and the Japanese anchovy (Engraulis japonicas). Canadian Journal of Zoology 96: 23–30.
Lynch AH, Kruger NJ, Hedges REM, McCullagh JSO. 2016. Variability in the carbon isotope composition of individual amino acids in plant proteins from different sources: 1 Leaves. Phytochemistry 125: 27–34.
Ma Y, Grimes V, Van Biesen G, Shi L, Chen K, Mannino MA, Fuller BT. 2021. Aminoisoscapes and palaeodiet reconstruction: New perspectives on millet-based diets in China using amino acid δ13C values. Journal of Archaeological Science 125: 105289.
Macho GA, Lee-Thorp JA. 2014. Niche partitioning in sympatric gorilla and Pan from Cameroon: Implications for life history strategies and for reconstructing the evolution of hominin life history. PLOS ONE 9: e102794.
Martin JE, Vance D, Balter V. 2015. Magnesium stable isotope ecology using mammal tooth enamel. Proceedings of the National Academy of Sciences 112: 430–435.
Matos MPV, Jackson GP. 2020. Compound-specific isotope analysis of human hair: Predicting behaviors and biometrics beyond dietary factors. Analytical Chemistry 92: 3014–3022.
Maurer A-F, Person A, Tütken T, Amblard-Pison S, Ségalen L. 2014. Bone diagenesis in arid environments: An intra-skeletal approach. Palaeogeography Palaeoclimatology Palaeoecology 416.
McClelland JW, Montoya JP. 2002. Trophic relationships and the nitrogen isotopic composition of amino acids in plankton. Ecology 83: 2173–2180.
McMahon KW, McCarthy MD. 2016. Embracing variability in amino acid δ15N fractionation: Mechanisms, implications, and applications for trophic ecology. Ecosphere 7: e01511.
McMahon KW, Fogel ML, Elsdon TS, Thorrold SR. 2010. Carbon isotope fractionation of amino acids in fish muscle reflects biosynthesis and isotopic routing from dietary protein. Journal of Animal Ecology 79: 1132–1141.
Meier-Augenstein W. 2018. Stable Isotope Forensics: An Introduction to the Forensic Application of Stable Isotope Analysis. Wiley.
Meier-Augenstein W, Schimmelmann A. 2019. A guide for proper utilisation of stable isotope reference materials. Isotopes in Environmental and Health Studies 55: 113–128.
Meltzer E, Schmidt HL. 1987. Carbon isotope effects on the pyruvate–dehydrogenase reaction and their importance for relative 13C depletion in lipids. Journal of Biological Chemistry 262: 8159–8164.
Metges CC. 2000. Contribution of microbial amino acids to amino acid homeostasis of the host. Journal of Nutrition 130: 18575–18645.
Moll RJ, Kilion AK, Hayward MW, Montgomery RA. 2021. A Framework for the Eltonian niche of humans. BioScience 71: 928–941.
Mor A, Pacheco A, Roberts C, Smith C. 2018. Pica 8: Refining dietary reconstruction through amino acid δ13C analysis of tendon collagen and hair keratin. Journal of Archaeological Science 93: 94–109.
Naito YI, Bocherens H, Chikaraishi Y, Drucker DG, Wiśniewska C, Yoneda M, Ohkouchi N. 2016a. An overview of methods used for the detection of aquatic resource consumption by humans: Compound-specific δ15N analysis of amino acids in archaeological materials. Journal of Archaeological Science Reports 6: 720–732.
Naito YI, Chikaraishi Y, Drucker DG, Ohkouchi N, Semal P, Wissing C, Bocherens H. 2016b. Ecological niche of Neanderthals from Spy Cave revealed by nitrogen isotope of individual amino acids in collagen. Journal of Human Evolution 93: 82–90.
Neps EPJG, Dejong CHC, Rensen SS. 2015. The role of microbial amino acid metabolism in host metabolism. Nutrients 7: 2930–2946.
Newsome SD, Feeler KL, Bradley CJ, Wolf C, Takacs-Vesbach C, Fogel ML. 2020. Isotopic and genetic methods reveal the role of the gut microbiome in mammalian host essential amino acid metabolism. Proceedings of the Royal Society B 287: 20192993.
Nielsen JM, Clare EL, Hayes N, Brett MT, Kratina P. 2018. Diet tracing in ecology: Method comparison and selection. Methods in Ecology and Evolution 9: 278–291.
O’Brien DM. 2015. Stable isotope ratios as biomarkers of diet for health research. Annual Review of Nutrition 35: 565–593.
O’Connell TC, Hedges RE, Healey M, Simpson AHRW. 2001. Isotopic comparison of hair, nail and bone: Modern analyses. Journal of Archaeological Science 28: 1247–1255.
O’Connell TC. 2017. “Trophic” and “source” amino acids in trophic estimation: A likely metabolic explanation. Oecologia 184: 317–326.
O’Connell TC, Collins MJ. 2018. Comment on “Ecological niche of Neanderthals from Spy Cave revealed by nitrogen isotopes of individual amino acids in collagen” [J. Hum. Evol. 93 (2016) 82–90]. Journal of Human Evolution 117: 53–55.
Oelze VM. 2016. Reconstructing temporal variation in great ape and other primate diets: A methodological framework for isotope analyses in hair. American Journal of Primatology 78: 1004–1016.
Ohkouchi N, et al. 2017. Advances in the application of amino acid nitrogen isotopic analysis in ecological and biogeochemical studies. Organic Geochemistry 113: 150–174.
Oliphant K, Allen-Vercoe E. 2019. Macronutrient metabolism by the human gut microbiome: Major fermentation by-products and their impact on host health. Microbiome 7: 91.
Paolini M, Ziller L, Laursen KH, Husted S, Camin F. 2015. Compound-specific δ15N and δ13C analyses of amino acids for potential discrimination between organically and conventionally grown wheat. Journal of Agricultural and Food Chemistry 63: 5841–5850.
Pate FD. 1994. Bone chemistry and paleodiet. Journal of Archaeological Science: Reports 6: 720–732.
Popp BN, Graham BS, Olson RJ, Lott MJ, López-Ibarra GA, Galván-Magaña F, Fry B. 2007. Insight into the trophic ecology of yellowfin tuna, Thunnus alalunga, from compound-specific nitrogen isotope analysis of proteinaceous amino acids. Terrestrial Ecology 1: 173–190.
trophic position estimation. Methods in Ecology and Evolution 12: 1750–1767.

Rapp Py-Daniel A. 2014. Bones: Preservation and conservation. Pages 985–989 in Smith C, ed. Encyclopedia of Global Archaeology. Springer.

Reeds PJ. 2000. Dispensable and indispensable amino acids for humans. Journal of Nutrition 130: 1835s–1840s.

Reed K. 2021. Food systems in archaeology. Examining production and consumption in the past. Archaeological Dialogues 28: 51–75.

Richards MP, Trinkaus E. 2009. Isotopic evidence for the diets of European Neanderthals and early modern humans. Proceedings of the National Academy of Sciences 106: 16034–16039.

Richards MP, Schulting RJ, Hedges REM. 2003. Sharp shift in diet at onset of Neolithic. Nature 425: 366–366.

Roberts P, Stewart BA. 2018. Defining the “generalist specialist” niche for Pleistocene Homo sapiens. Nature Human Behaviour 2: 542–550.

Roberts P, Fernandes R, Craig OE, Larsen T, Lucquin A, Swift J, Zech J. 2018. Calling all archaeologists: Guidelines for terminology, methodology, data handling, and reporting when undertaking and reviewing stable isotope applications in archaeology. Rapid Communications in Mass Spectrometry 32: 361–372.

Roeyer A, Duax V, Foureil F, Lécuyer C. 2017. Carbon, nitrogen and oxygen isotope fractionation during food cooking: Implications for the interpretation of the fossil human record. American Journal of Physical Anthropology 163: 759–771.

Sandberg PA, Sponheimer M, Lee-Thorp J, Van Gerven D. 2014. Intra-tooth stable isotope analysis of dentine: A step toward addressing selective mortality in the reconstruction of life history in the archaeological record. American Journal of Physical Anthropology 155: 281–293.

Schwartz-Narbonne R, Longstaffe FJ, Metcalfe JZ, Zazula G. 2015. Solving the woolly mammoth conundrum: Amino acid 15N-enrichment suggests a distinct forage or habitat. Scientific Reports 5: 9791.

Scott JH, O’Brien DM, Emerson D, Sun H, McDonald GD, Salgado A, Fogel ML. 2006. An examination of the carbon isotope effects associated with amino acid biosynthesis. Astrobiology 6: 867–880.

Sehrawat JS, Kaur J. 2017. Role of stable isotope analyses in reconstructing past life-histories and the provenancing human skeletal remains: A review. Anthropological Review 80: 243–258.

Smith BN, Epstein S. 1971. Two categories of 13C/12C ratios for higher plants. Plant Physiology 47: 380–384.

Smith C, Fuller B, Choy K, Richards M. 2009. A three-phase liquid chromatography–combustion–isotope ratio mass spectrometry for traceability and reporting when undertaking and reviewing stable isotope applications in archaeology. Rapid Communications in Mass Spectrometry 28: 4799.

Soncin S, et al. 2021. High-resolution dietary reconstruction of victims of the woolly mammoth conundrum: Amino acid 15N-enrichment suggests a distinct forage or habitat. Scientific Reports 5: 9791.

Styring AK, Fraser RA, Bogaard A, Evershed RP. 2014. Cereal grain, rachis and pulse seed amino acid δ15N values as indicators of plant nitrogen metabolism. Phytochemistry 97: 20–29.

Styring AK, Fraser RA, Arbogast R-M, Halstead P, Isaakidou V, Pearson JA, Schafer M, Triantaphyllou S, Valamoti SM, Wallace M. 2015. Refining human palaeodietary reconstruction using amino acid δ15N values of plants, animals and humans. Journal of Archaeological Science 33: 504–515.

Styring AK, Charles M, Fantone F, Hald MM, Mcalmon A, Meadow RH, Nicholls GK, Patel AK, Pitre MC, Smith A. 2017. Isotope evidence for agricultural extensification reveals how the world’s first cities were fed. Nature Plants 3: 17076.

Szpak P, Longstaffe FJ, Millaire J-F, White CD. 2014. Large variation in nitrogen isotopic composition of a fertilized legume. Journal of Archaeological Science 45: 72–79.

Takizawa Y, Chikaraisi Y. 2017. Change in the δ15N value of plant amino acids on the phenology of leaf flush and senescence. Researches in Organic Geochemistry 33: 1–6.

Takizawa Y, Dharampal PS, Steffan SA, Takano Y, Ohkouchi N, Chikaraisi Y. 2017. Intra-trophic isotopic discrimination of 15N/14N for amino acids in autotrophic Implications for nitrogen dynamics in ecological studies. Ecology and Evolution 7: 2916–2924.

Takizawa Y, Takano Y, Choi B, Dharampal PS, Steffan SA, Ogawa NO, Ohkouchi N, Chikaraisi Y. 2020. A new insight into isotopic fractionation associated with decarboxylation in organisms: Implications for amino acid isotope approaches in biogeochemistry. Progress in Earth and Planetary Science 7: 50.

Tieszen LL, Boutton TW, Tesdahl KG, Slade NA. 1983. Fractionation and turnover of stable carbon isotopes in animal-tissues: Implications for δ13C analysis of diet. Oecologia 57: 32–37.

Torrallardona D, Harris CI, Fuller MF. 2003. Pigs’ gastrointestinal microflora provide them with essential amino acids. Journal of Nutrition 133: 1127–1131.

Twiss K. 2012. The archaeology of food and social diversity. Journal of Archaeological Research 20: 357–395.

Ungar PS, Grine FE, Teaford MF. 2006. Diet in early Homo: A review of the evidence and a new model of adaptive versatility. Annual Review of Anthropology 35: 209–228.

Van Der Merwe NJ, Vogel JC. 1978. 13C content of human collagen as a measure of prehistoric diet in woodland North America. Nature 276: 815–816.

van der Merwe NJ, Medina E. 1991. The canopy effect, carbon isotope ratios and foodwebs in Amazonia. Journal of Archaeological Science 18: 249–259.

van Leeuwen KA, Premzler PD, Ryan D, Camin F. 2014. Gas chromatography–combustion–isotope ratio mass spectrometry for traceability and authenticity in foods and beverages. Comprehensive Reviews in Food Science and Food Safety 13: 814–837.

von Holstein IC, Penkman KE, Peacock EE, Collins MJ. 2014. Wet degradation of keratin proteins: Linking amino acid, elemental and isotopic composition. Rapid Communications in Mass Spectrometry 28: 2121–2133.

Walker PL, Deniro MJ. 1986. Stable nitrogen and carbon isotope ratios in bone collagen as indices of prehistoric dietary dependence on marine and terrestrial resources in Southern California. American Journal of Physical Anthropology 71: 51–61.

Wang C, et al. 2014. Aridity threshold in controlling ecosystem nitrogen cycling in arid and semi-arid grasslands. Nature Communications 4: 7499.

Wang YV, Wan AHL, Lock E-J, Andersen N, Winter-Schuh C, Larsen T. 2012. Know your fish: A novel compound-specific isotope approach for tracing wild and farmed salmon. Food Chemistry 256: 380–389.

Wang YV, Wan AHL, Krogdahl A, Johnson M, Larsen T. 2019. 13C values in keratin proteins: Linking amino acid, elemental and isotopic composition. Rapid Communications in Mass Spectrometry 28: 2121–2133.

Webb EC, Lewis J, Shain A, Kastrisianaki-Guyton E, Honch NV, Stewart A, Miller B, Tarlton J, Evershed RP. 2017. The influence of varying proportions of terrestrial and marine dietary protein on the stable carbon-isotope compositions of pig tissues from a controlled feeding experiment. STAR: Science and Technology of Archaeological Research 3: 36–52.
Weber D, Kexel H, Schmidt HL. 1997. 13C-pattern of natural glycerol: Origin and practical importance. Journal of Agricultural and Food Chemistry 45: 2042–2046.

Whiteman JP, Kim SL, McMahon KW, Koch PL, Newsome SD. 2018. Amino acid isotope discrimination factors for a carnivore: Physiological insights from leopard sharks and their diet. Oecologia 188: 977–989.

Whiteman JP, Elliott Smith EA, Besser AC, Newsome SD. 2019. A guide to using compound-specific stable isotope analysis to study the fates of molecules in organisms and ecosystems. Diversity 11: 8.

Whiteman JP, Rodriguez Curras M, Feeser KL, Newsome SD. 2021. Dietary protein content and digestibility influences discrimination of amino acid nitrogen isotope values in a terrestrial omnivorous mammal. Rapid Communications in Mass Spectrometry 35: e9073.

Wibowo MC, et al. 2021. Reconstruction of ancient microbial genomes from the human gut. Nature 594: 234–239.

Wiedenmair V, Scholl-Burgi S, Karall D, Huck CW. 2020. Amino acid profiles and compositions of different cultivars of Panicum miliaceum L. Chromatographia 83: 829–837.

Womack M, Rose WC. 1947. The role of proline, hydroxyproline, and glutamic acid in growth. Journal of Biological Chemistry 171: 37–50.

Wrangham R. 2017. Control of fire in the Paleolithic: Evaluating the cooking hypothesis. Current Anthropology 58: S303–S313.

Wrangham R, Conklin-Brittain N. 2003. Cooking as a biological trait. Comparative Biochemistry and Physiology A: Molecular and Integrative Physiology 136: 35–46.

Wyatt AS, Matsumoto R, Chikaraishi Y, Miyairi Y, Yokoyama Y, Sato K, Ohkouchi N, Nagata T. 2019. Enhancing insights into foraging specialization in the world’s largest fish using a multi-tissue, multi-isotope approach. Ecological Monographs 89: e01339.

Xu D, Liu J, Gu Y, Chen Y, Zhao C, Sun G, Ren Y, Li C, Xia B. 2021. Biosynthesis and isotopic routing of dietary protein by sea cucumber Apostichopus japonicus (Selenka): Evidence from compound-specific carbon stable isotope analysis. Journal of Agricultural and Food Chemistry 69: 14802–14809.

Yarnes C, Herszage J. 2017. The relative influence of derivatization and normalization procedures on the compound-specific stable isotope analysis of nitrogen in amino acids. Rapid Communications in Mass Spectrometry 31: 693–704.

Yoshito C, Yuichiro K, Nanako OO, Hiroshi K, Naohiko O. 2007. Metabolic control of nitrogen isotope composition of amino acids in macroalgae and gastropods: Implications for aquatic food web studies. Marine Ecology Progress Series 342: 85–90.

Yu T-Y, Morton JD, Clerens S, Dyer JM. 2017. Cooking-induced protein modifications in meat. Comprehensive Reviews in Food Science and Food Safety 16: 141–159.

Yun HY, et al. 2020. The Carbon isotope ratios of serum amino acids in combination with participant characteristics can be used to estimate added sugar intake in a controlled feeding study of US postmenopausal women. Journal of Nutrition 150: 2764–2771.

Zink KD, Lieberman DEJN. 2016. Impact of meat and Lower Palaeolithic food processing techniques on chewing in humans. Nature 531: 500–503.

Thomas Larsen (larsen@shh.mpg.de), Ricardo Fernandes, Yiming V. Wang, and Patrick Roberts are affiliated with the Department of Archaeology at the Max Planck Institute for the Science of Human History, in Jena, Germany. Ricardo Fernandes is affiliated with the School of Archaeology at the University of Oxford, in Oxford, England, in the United Kingdom, and with the Faculty of Arts at Masaryk University, in the Czech Republic. Patrick Roberts is affiliated with the School of Social Sciences at the University of Queensland, in St Lucia, Queensland, Australia.