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The power and pitfalls of amino acid carbon stable isotopes for tracing origin and use of basal resources in food webs

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OVERVIEW

Abstract

Natural and anthropogenic stressors alter the composition, biomass, and nutritional quality of primary producers and microorganisms, the basal organisms that synthesise the biomolecules essential for metazoan growth and survival (i.e. basal resources). Traditional biomarkers have provided valuable insight into the spatiotemporal dynamics of basal resource use, but lack specificity in identifying multiple basal organisms, can be confounded by environmental and physiological processes, and do not always preserve in tissues over long timescales. Carbon stable isotope ratios of essential amino acids (δ^{13}C-EAA) show remarkable promise in identifying and distinguishing clades of basal organisms with unique δ^{13}C-EAA fingerprints that are independent of trophic processing and environmental variability, providing unparalleled potential in their application. Understanding the biochemical processes that underpin δ^{13}C-AA data is crucial however for holistic and robust inferences in ecological applications. This comprehensive methodological review conceptualises for the first time these mechanistic underpinnings that drive δ^{13}C-EAA fingerprints among basal organisms and incorporate δ^{13}C values of non-essential amino acids that are generally overlooked in ecological studies, despite the gain of metabolic information. We conduct meta-analyses of published data to test hypothesised AA-specific isotope fractionations among basal organism clades, demonstrating it is phenylalanine that separates vascular plant δ^{13}C-EAA fingerprints, which strongly covary with their phylogeny. We further explore the utility of non-essential AAs in separating dietary protein sources of archaeological humans, showing the differences in metabolic information contained within different NEAAs. By scrutinising the many methodologies that are applied in the field, we highlight the absence of standardised analytical protocols, particularly in sample pretreatments leading to biases; inappropriate use of statistical methods; and reliance on unsuitable training data. To unlock the full potential of δ^{13}C-EAA fingerprints, we provide in-depth explanations on knowledge gaps, pitfalls, and optimal practices in this complex but powerful approach for assessing ecosystem change across spatiotemporal scales.

1. Introduction

Food webs are increasingly impacted by anthropogenic stressors such as accelerated climate change, biodiversity loss, habitat destruction, and pollution (Hoegh-Guldberg and Bruno 2010, Blanchard et al. 2012, Kędra et al. 2015). These stressors can disrupt the natural processes and environmental cycles that determine the timing, location, and magnitude of primary producer and microbe productivity (Eker-Develi et al. 2006, Vining et al. 2022). Higher trophic-level organisms rely on suites of biomolecules - referred to as basal resources - synthesised by primary producers and microbes (basal organisms). Changes in the abundance and nutritional quality of basal organisms can therefore have far-reaching implications for the dynamics, structure, functioning and stability of food webs (Nakazawa 2015, Svanbäck et al. 2015, Kortsch et al. 2015). However, changes in basal organisms and the assimilation of their basal resources by higher trophic levels occur over intricate spatiotemporal scales and on fine-scale taxonomic levels (Raubenheimer et al. 2012, McMeans et al. 2015, Chidawanyika et al. 2019). A precise and consistent approach to tracing the origin of basal resources in food webs therefore facilitates assessing the vulnerability of species, food webs and entire ecosystems to environmental change (Moloney et al. 2011).

Among the analytical approaches for tracing trophic transfers (e.g. gut content analysis, metabarcoding, fatty acid profiling and stable isotope analyses), measuring carbon stable isotope compositions has emerged as a standard approach for tracing the assimilation of basal organisms to higher trophic levels. The relative abundance of heavy (^{13}C) to light (^{12}C) carbon isotopes, normalised to the international standard (Vienna Pee Dee Belemnite, VPDB) and expressed as $\delta^{13}C$ per mille (‰) values, are measured within all carbon-containing biomolecules, i.e. bulk. The values of consumer tissues are then compared to their potential basal resources. $\delta^{13}C$ values are highly suited to trace basal resources because carbon is abundant, ubiquitous, and $\delta^{13}C$ values of basal resources are often habitat or taxon specific. However, bulk $\delta^{13}C$ values of basal resources can vary substantially with the environment (Peterson and Fry 1987, Casey and Post 2011, Magozzi et al. 2017), which adds complexity to reconstructing basal resource use. Moreover, bulk $\delta^{13}C$ values, as only a single tracer, have a limited ability to distinguish between the multitude of basal resources in a given ecosystem and contributions from microorganisms are frequently underappreciated due to the logistical challenge of sampling them in situ (Casey and Post 2011).

To address the constraints of bulk tissue analysis, researchers increasingly analyse $\delta^{13}C$ values of individual biomolecules (Nielsen et al. 2017, Ruess and Müller-Navarra 2019). Basal organisms fix external carbon to synthesise their own biomolecules. Following ingestion, digestion, and absorption,

these biomolecules are assimilated into consumer tissues with minimal modification of their core structure; catabolized for energy; or used in the synthesis of new biomolecules (Boecklen et al. 2011). Individual fatty acids have proven valuable for tracing basal resources to consumers in modern food webs (Burian et al. 2020). However, fatty acids are less suited for past basal resource use reconstructions because of their low concentration and degradation in most structural tissues that persist in palaeoecological records (Geigl et al. 2004). The $\delta^{13}C$ values of the 20 proteinogenic amino acids (AAs) show considerable promise to identify specific basal resources from primary producers and microbial organisms. $\delta^{13}C$-AA values can trace their carbon transfer irrespective of environmental conditions (Larsen et al. 2009, Elliott Smith et al. 2022, Vane et al. 2023), serving as powerful spatiotemporal tracers of basal resource use. As AAs exhibit stable preservation in fossilised biogenic carbonates such as dinosaur eggshells, coral skeletons, and fish otoliths or other preserved structural tissues (Abelson 1954, Hare et al. 1991, Mora et al. 2018, Ma et al. 2021), $\delta^{13}C$-AA values allow for detailed retrospective inferences of basal resource use by animals across contemporary, paleontological, and geological records.

Animals can synthesise 11 of the 20 proteinogenic AAs de novo. The non-synthesizable AAs, or essential amino acids (EAAs, Wu et al. 2014), must be acquired from the diet or supplemented from the gut microflora. The contribution from gut microflora is thought to be minor for most healthy animals feeding on nutritionally adequate diets (Fuller and Reeds, 1998). Since EAAs are routed directly from dietary proteins, their tissue-diet $\delta^{13}C$ offsets are negligible (McMahon et al. 2010, 2015b, Takizawa et al. 2017, Wang et al. 2019a). EAAs have a powerful source diagnostic potential to trace basal resource transfer to animal biomass as broad taxonomic groups such as algae, bacteria, fungi, and vascular plants each have characteristic $\delta^{13}C$-EAA patterns: the relative differences in $\delta^{13}C$ values between EAAs (Scott et al. 2006, Larsen et al. 2013, 2015, Lynch et al. 2016, Elliott Smith et al. 2018, 2022, Stahl et al. 2023). Distinct $\delta^{13}C$-EAA patterns among basal organisms that remain largely consistent across variable physiochemical conditions and through time have been typically referred to as $\delta^{13}C$-EAA fingerprints (Larsen et al. 2009). For the metazoan-synthesizable AAs, commonly termed the non-essential amino acids (NEAAs), animals may rely both on dietary sources and de novo synthesis. However, most NEAAs can be considered conditionally essential for metazoans, particularly during stages of rapid growth when the rate of utilisation outpaces the rate of synthesis, constraining normal physiological and metabolic processes without dietary supplementation (Wu 2009, Eisert 2011, Hou et al. 2015).

Despite the increasing use of δ^{13}C-AA values in archaeological and ecological food web studies, appreciation of the mechanistic processes that underpin δ^{13}C-AA values and the δ^{13}C-EAA fingerprinting approach is limited (Nielsen et al. 2017, Whiteman et al. 2019, Ruess and Müller-Navarra 2019, Yun et al. 2022). Moreover, the wide variety of analytical and statistical methodologies currently in use may be inhibiting robust applications, and the complementary metabolic and nutritional information concealed in consumer δ^{13}C values of NEAAs is generally overlooked (δ^{13}C-NEAA, McMahon et al. 2015b). To progress the field and unlock the full potential of δ^{13}C-AA data, a solid mechanistic understanding of the underlying biochemistry is required, along with identifying pitfalls and establishing consistent methodologies. This review provides the first comprehensive framework of the application of carbon isotopes in AAs for inferring the origin and use of basal resources within food webs. By covering the full process from biochemical mechanisms and sampling to analysis and interpretation, we identify potential pitfalls and highlight areas for further investigation. We build a conceptual framework for understanding the factors influencing δ^{13}C-AA values and establish a standardised terminology in the field (see Table 1). Postulating on the specific mechanisms that give rise to the discriminatory power of δ^{13}C-EAA patterns, we explore these hypotheses using a global data compilation. We expand our framework to incorporate the additional complexities of NEAAs, and demonstrate how inclusion of δ^{13}C-NEAA values can provide additional insight into spatiotemporal resource use and individual metabolisms. Emphasising the importance of accurate measurements, we highlight best practices within analytical protocols, and address the critical issue of correctly applying mixing models for robust quantification of basal resource use by consumers. With proper use of the wealth of information provided by δ^{13}C-AA values, the specific drivers of food web productivity and their spatiotemporal dynamics can be explored, providing a powerful and currently unprecedented way to assess changing ecosystems.

Table 1. Glossary of terminology and associated quantitative measures used in carbon stable isotope analysis of amino acids

Terminology	Definition
Amino acids - Essential (EAA)	Proteinogenic amino acids that cannot be synthesised de novo by metazoans: histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine.
Amino acids - Non-essential (NEAA)	Proteinogenic amino acids that can be synthesised de novo by (most) metazoans: alanine, arginine, asparagine, aspartic acid,

	cysteine, glutamic acid, glutamine, glycine, proline, serine, and tyrosine.
Auxotrophs	Organisms that lack the capability to synthesise particular biomolecules de novo (applied here specifically to EAA synthesis, antonym of prototrophs).
Basal organisms	Primary producers and microbes that synthesise suites of biomolecules de novo from externally sourced (in)organic carbon (prototrophs), considered to be the base of food webs.
Basal resources	The suites of biomolecules (focusing on AAs in this review) synthesised de novo by basal organisms and assimilated by consumers for normal physiological functioning.
Basal resource use reconstruction	Estimating the proportions of basal resources synthesised by specific basal organism groups or clades that have been assimilated into consumer tissues
Facultative EAA-prototrophs	Organisms that can synthesise EAAs de novo, but have the capacity to assimilate externally derived EAAs for normal metabolic functioning.
Obligate EAA-prototrophs	Autotrophs that solely synthesise the EAAs they need solely from simple inorganic carbon sources fixed through photo- or chemosynthesis.
Training data	A compilation of δ^{13}C-AA values, previously measured external to the current study, used to characterise basal resources in a study system.
Trophic Discrimination Factor (TDF)	The isotopic offset between a consumer tissue and the assimilated diet, capturing isotope fractionations due to metabolic processes.
Quantitative Terminology	**Definition**
Acquired ^{13}C-AA data	Ratios of ^{13}C to ^{12}C in individual amino acids, uncorrected for measurement biases and not standardised to VPDB.
Measured δ^{13}C-AA values	The VPDB standardised (δ) carbon stable isotope values of AAs, corrected for measurement protocol biases, that are physically quantified in a sample.
Baseline δ^{13}C-AA values	The measured δ^{13}C values of AAs in basal organism tissues.
δ^{13}C-AA pattern	The relative offsets between individual δ^{13}C-AA values within a sample. For basal resource use reconstructions, typically only the offsets between EAA are used (δ^{13}C-EAA patterns).
δ^{13}C-EAA fingerprint	The minimum δ^{13}C-EAA pattern space that is solely occupied by a group or collection of similar basal organisms and encompasses the intragroup variability in δ^{13}C-EAA patterns expressed by those organisms.

2. Factors shaping amino acid $\delta^{13}C$ values in basal organisms

A thorough knowledge of the metabolic pathways that shape intermolecular ^{13}C distributions is essential for understanding how and why $\delta^{13}C$-AA patterns in basal organisms vary across the diversity of life. The rigorous application of $\delta^{13}C$-AA patterns therefore requires the development of a mechanistic framework, which has so far been lacking (Hayes 2001). While variations between different biosynthetic pathways have been acknowledged as a key driver in diverging $\delta^{13}C$-AA patterns among taxa (Larsen et al. 2009), ecological applications of $\delta^{13}C$-AA patterns are still mostly driven by phenomenological observations (e.g. Stahl et al. 2023). By conceptualising the processes that give rise to $\delta^{13}C$-AA values in basal organisms, we highlight how specific mechanisms can dominate the relative $\delta^{13}C$ offsets of certain AAs, underpinning the distinction of $\delta^{13}C$-AA patterns between taxa. Explicit definitions for $\delta^{13}C$-AA terminology are proposed to establish an unambiguous basis for subsequent discussions and interpretations. By establishing this foundation, we lay the groundwork for further developing the applications of $\delta^{13}C$-AA in ecological research.

2.1. Conceptualising amino acid $\delta^{13}C$ values in basal organisms

Basal organisms are those that can synthesise basal resources de novo, here specifically considered the full suite of 20 proteinogenic AAs. The ability to synthesise particular biomolecules, such as AAs, is termed prototrophy (the inability being auxotrophy). The majority of basal organisms, the AA prototrophs, are autotrophic, relying on photo- or chemosynthesis to fix inorganic carbon for the synthesis of all their biomolecules, including AAs. However, some basal organisms such as fungi and bacteria are heterotrophic and obtain organic carbon for both chemical energy and de novo synthesis of biomolecules. The pathways from external sources of (in)organic carbon to intracellular AA synthesis can be generalised into two broad categories. The first is the collection of processes involved in the uptake and conversion of external carbon to internal pools of common precursor molecules, which we refer to as carbon acquisition. The second is the biochemical reactions that synthesise the specific AAs from these precursors (Figure 1). Mass-dependent kinetic isotope fractionations associated with these biosynthetic pathways result in stepwise changes in relative isotopic ratios as either lighter or heavier carbon atoms diffuse passively, are actively transported, or react in anabolic and catabolic processes at different rates (Figure 2, Hayes 2001, Fry 2006). The carbon isotope composition of basal resources

therefore reflects the summation of all stepwise fractionations from the isotopic composition of the initial carbon pool to the synthesis of AAs.

Synthesis pathways among AAs are unique, and therefore comprise different summations of kinetic isotope fractionations (Appendix S1: figures S1a,b). This contrasts with carbon acquisition where total isotopic fractionation will be reflected relatively equally across AAs due to common pools of precursor molecules. Basal organisms use various sources of external carbon that have inherent carbon isotope compositions. Rates of diffusion, transport, and chemical reactions depend on various environmental factors that cause isotopic fractionation during carbon acquisition. The isotopic composition of external carbon also depends on various kinetic processes, and therefore will also vary with environmental conditions. Taken together, the δ^{13}C value of an AA in a basal organism can be broadly formulated as:

$$\delta^{13}C_{AA} \sim \delta^{13}C_{Ext.} + Env. \times Ext. + Acq. + Env. \times Acq. + Synth_{AA} \quad [1]$$

Where $\delta^{13}C_{AA}$ is given by the δ^{13}C value of the external carbon, *Ext.*; plus any modifications to this value due to environmental effects, *Env.,* dependent on the nature of the external carbon; plus the summed fractionations associated with carbon acquisition, *Acq.*; plus any modifications due to environmental effects on the physiology associated with carbon acquisition fractionation; plus the summed fractionation associated with synthesis pathway, *Synth.,* which is AA specific (visualised in Figure 1). Environmental gradients can modify the specific fractionations associated with each AA synthesis pathway, however these differences will likely be very small compared to the overall average effect of the environment on physiology and therefore carbon acquisition (Stahl et al. 2023, Larsen et al. 2015, Figure 3a,b). From [1], the measured δ^{13}C values of AAs in basal organisms therefore depend on the carbon source, the environment and phylogeny (via their fixation and synthesis pathways). This aligns with the concept of multiple isotopic baselines in bulk stable isotope approaches that characterise the base of the food web contextualised with in situ environmental conditions for different production sources (e.g. Docmac et al. 2017, Søreide et al. 2006). We therefore define measured δ^{13}C-AA values in basal organisms as baseline δ^{13}C-AA values (Figure 3a).

If we consider the isotopic fractionations of AA biosynthesis as relative differences (i.e. $Synth_{AA}$ averages to zero) then they can be regarded as a relative ordination centred on their mean value. We denote this relative ordination of $Synth_{AA}$ specifically as (1|AA) in Figure 1. Conceptually, this means that any

non-zero average fractionation across AA biosynthesis pathways will be incorporated as part of the acquisition term, but has the advantage that the collection of AA biosynthesis fractionations can be considered as a relative ordination that is imposed onto the average baseline bulk (protein) δ^{13}C value of the basal organism:

$$Average\ \delta^{13}C_{AA} = \frac{1}{n}\sum_{i=1}^{n} \delta^{13}C_{AA} \sim \delta^{13}C_{Ext.} + Env. \times Ext. + Acq. + Env. \times Acq. \quad [2]$$

where *n* is the number of AAs. It follows that the ordination can be determined as:

$$(1|AA) = Baseline\ \delta^{13}C_{AA} - Average\ \delta^{13}C_{AA} = \delta^{13}C_{AA} - \frac{1}{n}\sum_{i=1}^{n} \delta^{13}C_{AA} \quad [3]$$

The relative offset for each AA is simply the individual baseline δ^{13}C-AA value minus the mean-centred δ^{13}C-AA value of the basal organism (the non-weighted, within-sample average δ^{13}C-AA value), which we define as the δ^{13}C-AA pattern (Figure 3b). Expressing δ^{13}C-AA patterns via mean-centring is the standard approach first introduced by Larsen et al. (2009, denoted as $\delta^{13}C_N$). However, an important constraint is that changes in the combination of AAs results in changes in the absolute offsets in the expressed δ^{13}C-AA pattern, although not the pairwise AA differences.

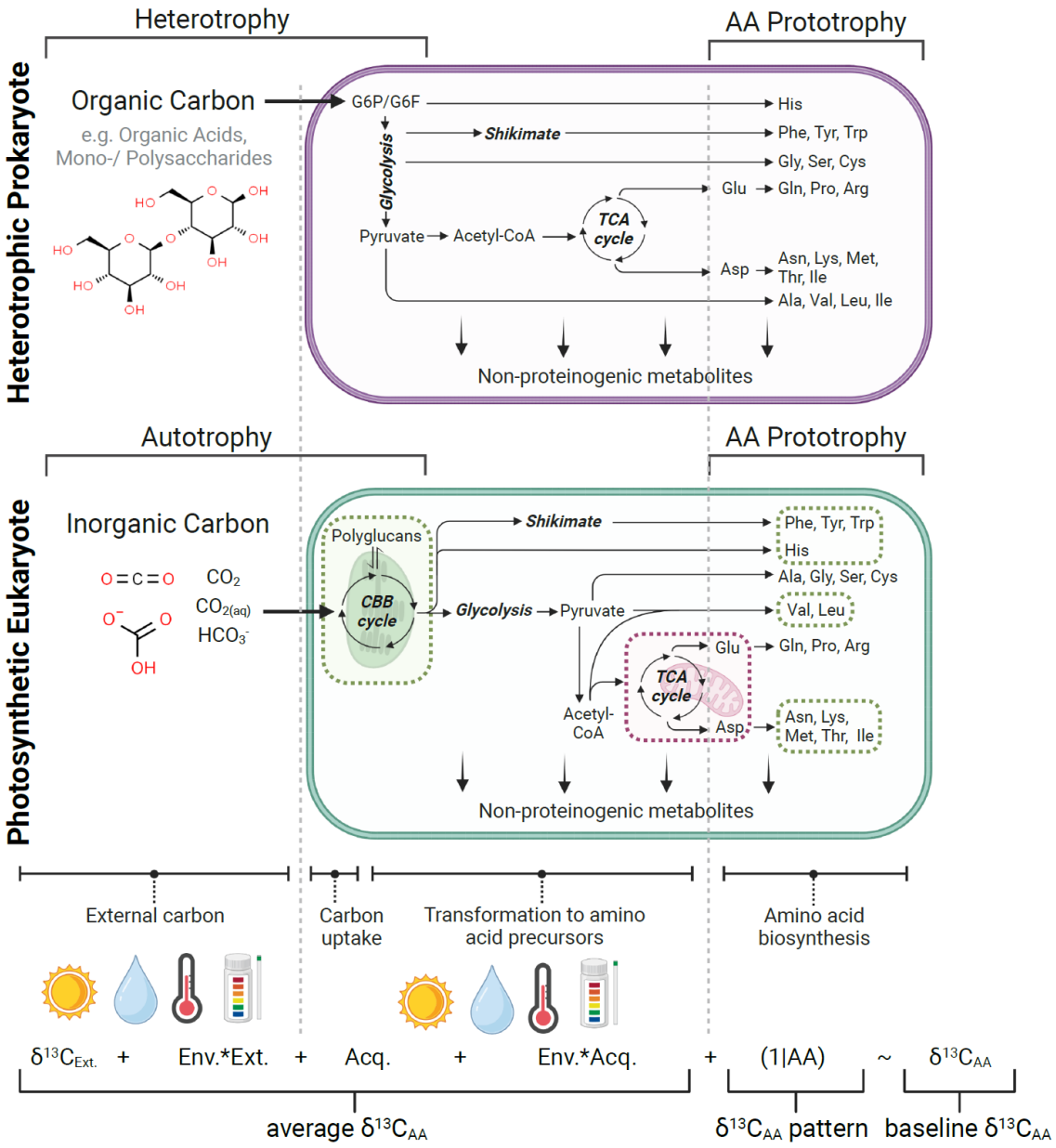

Figure 1. Schematic representation of the sources, processes, and environmental effects that contribute to the $\delta^{13}C$ values of synthesised AAs (basal resources) in two prototrophs - a heterotrophic prokaryote and a photosynthetic eukaryote. Within the eukaryotic cell, membrane bound organelles are signified by rectangles with dashed lines: mitochondria (red), and plastids (green) including the chloroplast. Metabolic pathways are based on Chen et al. (2018), and Gupta and Gupta (2021). Detailed metabolic networks are provided in Appendix S1: figures S1a,b. Abbreviations: Ala, alanine; Asn, asparagine; Asp, Asparagine; CBB, Calvin-Benson-Bassham; Cys, cysteine; F6P, Fructose-6 phosphate; G6P, Glucose-6 phosphate; Gly, glycine; Gln, glutamine; Glu, glutamic acid; His, histidine; Ile, isoleucine; Leu, leucine, Lys, lysine; Met, methionine; Phe, phenylalanine; Pro, proline; Ser, serine; TCA, Tricarboxylic acid; Trp, tryptophan; Tyr, tyrosine; Val, valine. The illustration was created with BioRender.com.

2.2. Isotope fractionations in metabolic networks

While many processes affect measured δ^{13}C-AA values in basal resources, differences in the δ^{13}C-AA patterns among basal organisms should conceptually arise solely from variations in summed stepwise isotope fractionations associated with the AA biosynthesis pathways (Figure 1). Figure 2 shows a simple hypothetical biochemical network, emphasising some of the diverse processes that transpire during biosynthesis. δ^{13}C values of synthesised biomolecules are underpinned by two factors: the kinetic isotopic effect of the step processes, and the relative flow rates of reactant replenishments and product removals (Hayes 2001). Consequently, three distinct mechanisms can alter δ^{13}C-AA offsets and hence the δ^{13}C-AA patterns in basal organisms: distinct biosynthesis pathways for the same AA; different modulating enzymes for individual steps within AA pathways; and different flows of pathway reactants and products, including the synthesised AA product.

For many AAs, multiple synthesis pathways exist across different basal organism taxa. As different synthesis pathways comprise different steps (e.g. the synthesis of E from B with either C or D as an intermediate in Figure 2), they result in different δ^{13}C offsets for their respective AAs across taxa. A notable example are the three aromatic AAs that are synthesised from the shikimate pathway using the chorismate precursor (Figure 1). Two pathways exist for phenylalanine that differ in the final two reaction steps. Fungi and bacteria use phenylpyruvate as an intermediate that is converted to phenylalanine via the transfer of an amine group. In contrast, plants and algae first synthesise the non-proteinogenic AA arogenate, then modify the side chain to produce phenylalanine. Tyrosine follows a similar path, with plants and algae using arogenate as an intermediate while bacteria and fungi form tyrosine from hydroxy-phenylpyruvate. The third AA synthesised from chorismate is tryptophan, a biochemically complex and expensive pathway that has been evolutionary conserved, involving homologous reaction steps across the three domains of life (KEGG PATHWAY 2013). Among the aromatic AAs, it is expected that phenylalanine and tyrosine δ^{13}C offsets in plants and algae may differ from bacteria and fungi, but not for tryptophan. For biochemically simple AAs such as lysine, separate anabolic synthesis routes exist: the diaminopimelic acid pathway is used predominantly by algae and plants, while the α-aminoadipic acid pathway is predominantly used by fungi, with bacteria and archaea utilising both pathways (Velasco et al. 2002). Within these two broad routes, six major pathways have emerged among different taxa, giving lysine a particularly high diagnostic potential in δ^{13}C-AA offsets (Larsen et al. 2009).

Within seemingly identical biosynthesis pathways, individual steps can be modulated by different enzymes (enzyme-A vs enzyme-B in Figure 2). Differing enzyme structures and catalytic efficiencies may cause variations in the kinetic isotopic effects during individual steps of biosynthetic pathways. A prime example of enzymatic fractionation differences occurs in Rubisco, the enzyme that fixes CO_2 in the Calvin-Benson-Bassham cycle (but is not involved in AA synthesis, Figure 1). Plant and algae Rubisco (form I) has a larger fractionation (~30‰) than prokaryotic Rubisco (form II; ~22‰, Guy et al. 1993, Hayes 2001). Across the AA synthesis pathways, diverse classes of enzymes may be used that are general or reactant-specific, and therefore vary in their isotopic fractionations, contributing to distinct δ^{13}C-AA patterns among basal organisms. However, ^{13}C kinetic isotope fractionation primarily occurs when the rate limitation of the catalysing enzyme consists of bond cleaving, formation, or transfers involving carbon atoms. Consequently, not all catalysed processes will result in an observable ^{13}C fractionation even if the overall reaction step involves the breaking or formation of carbon-linked bonds. For example, the synthesis of glutamine from glutamate, a process where an amine group is bound to the end carbon atom of the glutamate side chain, does not result in ^{13}C fractionation. This is because the rate limitation occurs during the amine-deprotonation and release of glutamine from the catalysing enzyme, which involves only nitrogen and hydrogen atoms (Mauve et al. 2016). To accurately predict potential differences in fractionation rates for specific pathway steps, detailed information about reaction kinetics is required.

As AA biosynthesis pathways are embedded within larger interconnected metabolic networks, differences in the upstream supply of reactants and downstream demands of products can result in asynchronous flow rates between pathway steps. This could lead to differential isotope fractionation if flow rate differences are substantial. One key mechanism underpinning flow rates is intracellular compartmentalisation (e.g. the movements of A_1, E_1 and E_2 in Figure 2), with prokaryotes carrying out AA synthesis in the cytoplasm, whereas eukaryotes additionally synthesise AAs in organelles - involving the active movement of molecules across intracellular membranes. Clade-specific demands for proteinogenic AAs as precursors for secondary metabolites, energy-yielding substrates, and metabolic donors (Appendix S1: figure S1a,b,c) may influence flow rates (e.g the downstream branching of E_3 in Figure 2: increasing demand for F_1 will reduce the flow rate to E_4). In higher plants, the synthesis of alkaloid compounds relies on several nitrogenous precursors such as phenylalanine, lysine, and histidine (Aniszewski 2007). In comparison, algae have very low concentrations of alkaloids and therefore lack this downstream AA demand (Güven et al. 2010). Similarly, the biosynthesis of phenylpropanoids, the backbone of lignin in vascular plants, uses phenylalanine as a precursor (Vanholme et al. 2010). If such

supply and demand flows of AAs are substantial, lineage specific, and consistent then systematic differences in δ^{13}C-AA patterns emerge.

Differences in the synthesis pathways, modulating enzymes, and flow rates between basal organisms that result in distinct δ^{13}C-AA patterns are likely expressed at different taxonomic levels. AA synthesis pathways primarily vary among the broadest taxonomic levels due to the extensive suites of functional genes required. The major eukaryotic clades of plants, algae and fungi typically only possess a single synthesis pathway for each AA, with plants and algae often sharing the same pathway. In contrast, multiple pathways for some AAs are found within bacteria (e.g. 5 of the 6 lysine synthesis pathways) and to a lesser extent Archaea, following their greater genetic diversity. It therefore can be expected that the prokaryotic clades express greater variability in their δ^{13}C-AA patterns compared to eukaryotes. Variation in genetically encoded enzyme structures can occur at lower levels of phylogeny, as they constitute more limited genetic differences. Enzyme mediated fractionations of ^{13}C can vary substantially (Hayes 2001), but this variation depends on whether the rate limitation of the specific reaction being mediated involves carbon atoms (Mauve et al. 2016). Therefore, differences in enzymes may not always result in δ^{13}C-AA pattern differences between taxa. Differential flow rates in AA biosynthesis pathways are the most flexible mechanism through which isotope fractionations may differ, as they can be altered by regulating gene expression (e.g. increasing the number of transmembrane protein channels), and may occur across the different levels of phylogeny. Substantial demands for AAs to synthesise secondary metabolites that constitute significant proportions of organismal biomass may dominate trends in δ^{13}C-AA patterns between phenotypes. Therefore, there is significant potential for δ^{13}C-AA patterns to be diagnostic of the origin of basal resources from broad to fine levels of phylogeny in basal organisms.

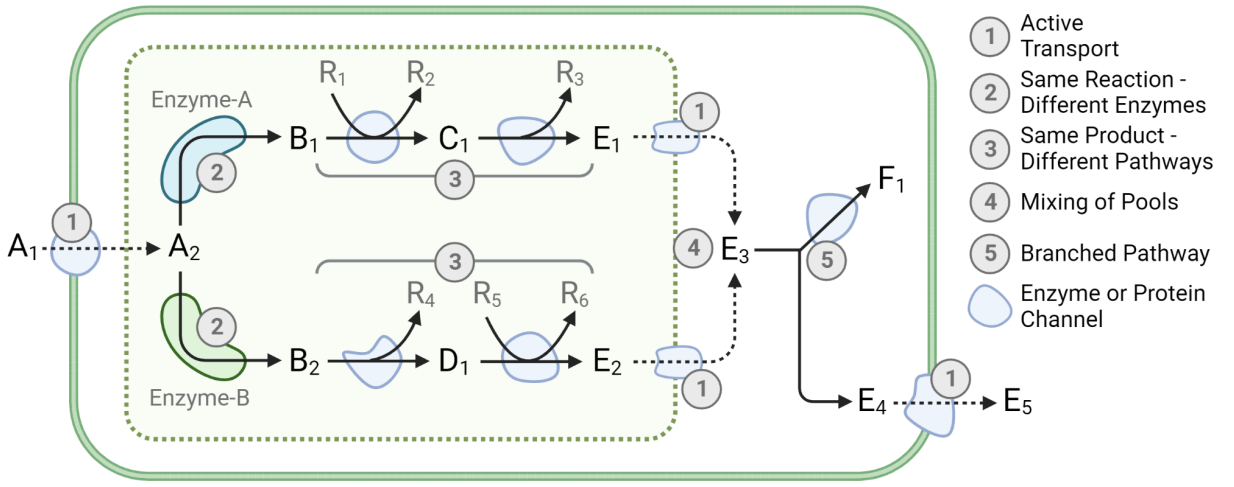

Figure 2. A simple, hypothetical biochemical network within a eukaryotic cell, highlighting different processes that lead to isotopic differences in synthesised biomolecules. Focal compounds are denoted by capital letters with numerical subscripts distinguishing between different pools that may differ in isotopic composition. Secondary compounds are denoted as R_n in grey. Arrows denote the flow of a compound from one pool to another, with solid arrows indicating a chemical reaction and dashed arrows a movement of molecules. This illustration was created with BioRender.com.

3. Discriminating basal resources with δ^{13}C-EAA fingerprints

Although all AAs and their δ^{13}C values can be used to distinguish between basal resources, the nine canonical EAAs are the most valuable indicators when reconstructing basal resource use in consumers. The stability of δ^{13}C-EAA values during trophic transfer due to the direct routing of EAAs means that their relative offsets, and hence the δ^{13}C-EAA patterns of basal resources, the EAA subset of the δ^{13}C-AA patterns, are also preserved (McMahon et al. 2010, 2015b, Liu et al. 2018, Wang et al. 2019a). Published δ^{13}C-EAA patterns have already shown the unique ability to discriminate groups of basal organisms, yet offer limited understanding of the underlying processes and potential taxonomic specificity. To develop this understanding, we build upon our mechanistic framework laid out in section 2 by compiling and exploring published δ^{13}C-EAA values of basal organisms at varying levels of phylogeny. We discuss the potential for direct assimilation of EAAs from the environment by some basal organisms. Based on these mechanistic considerations, we refine the definition of δ^{13}C-EAA fingerprints and outline their optimal characterisation.

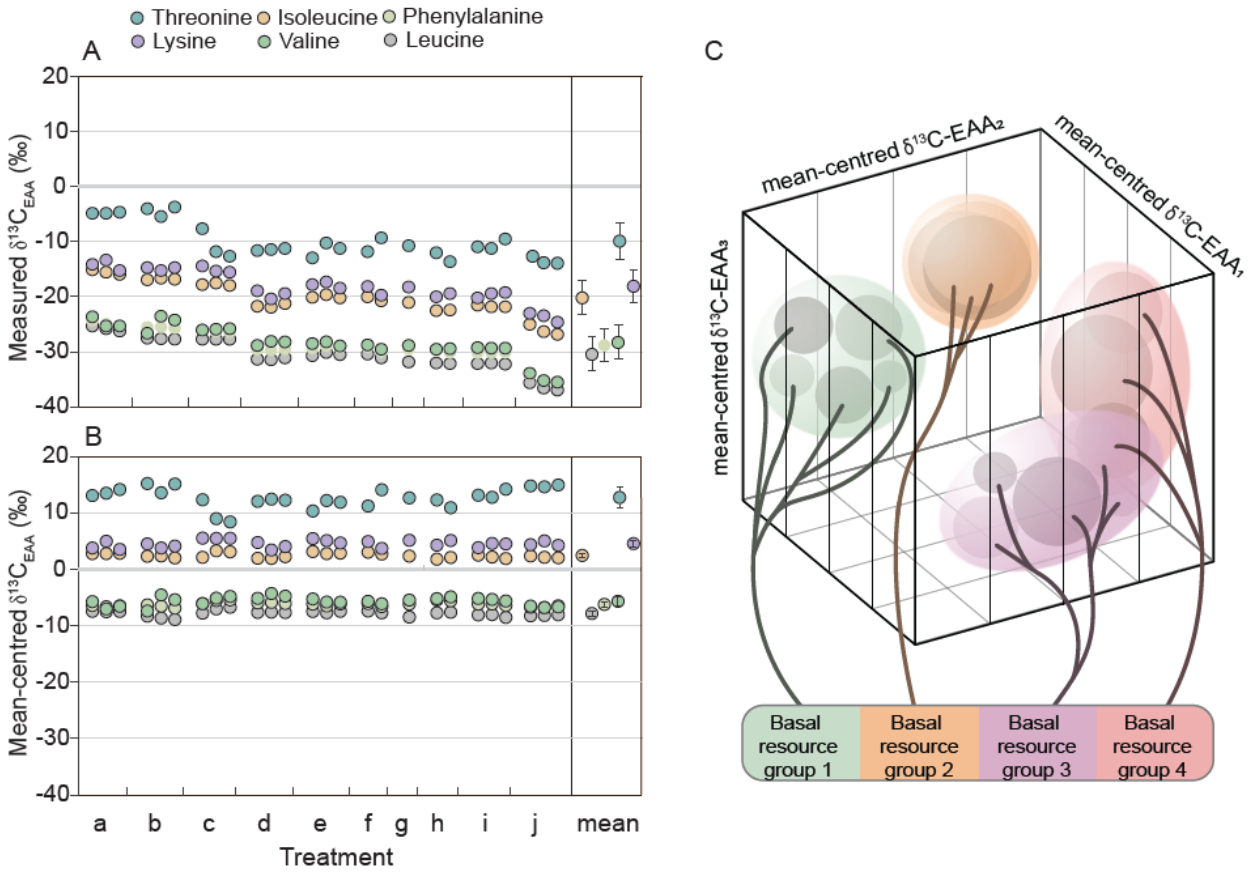

Figure 3. The progression from baseline δ^{13}C-EAA values (A) to δ^{13}C-EAA patterns (B) to δ^{13}C-EAA fingerprints (C). A - The measured δ^{13}C values of six EAAs in the marine diatom *Thalassiosira weissflogii,* cultured under different conditions, show how different environmental conditions influence baseline δ^{13}C-EAA values : a) 27°C, b) 18°C, c) High pH, d) Control, e) UV filter, f) No UV filter, g) Low irradiance, h) High irradiance, i) Low pH, j) Low salinity (mean and standard deviation for each EAA across treatments given on the right, data from Larsen et al. 2015). B - By mean-centring the baseline δ^{13}C values within samples, the consistency in δ^{13}C-EAA patterns of *T. weissflogii* across environments becomes apparent. C - Comparing the δ^{13}C-EAA patterns of different basal resource groups determines whether the δ^{13}C-EAA patterns constitute δ^{13}C-EAA fingerprints within a study system (illustrated with 3 EAAs). A basal resource group has a δ^{13}C-EAA fingerprint when that group solely occupies its δ^{13}C-EAA pattern space, e.g. groups 1 and 2. The specificity of the δ^{13}C-EAA fingerprint can be high if subgroups of the basal resource (illustrated by branches) occupy unique subspaces within their overall fingerprint, ca. group 1 with group 2. δ^{13}C-EAA patterns are not considered δ^{13}C-EAA fingerprints if different basal resource groups exhibit overlap in δ^{13}C-EAA pattern space, e.g. groups 3 and 4.

3.1. The diagnostic potential of δ^{13}C-EAA patterns among basal resources

Five EAAs typically reported across studies are leucine, isoleucine, phenylalanine, threonine and valine. Of these five, all except phenylalanine share a common biosynthesis pathway across the domains of life: phenylalanine has two common pathways that are split between plants and algae, and bacteria and fungi (section 2.2, Appendix S1: figure S1a,b). Although some bacteria and plants can use alternative isoleucine synthesis pathways (Sugimoto et al. 2021), the rarity of these pathways is unlikely to cause

divergence in clade specific isoleucine $\delta^{13}C$ offsets. Bacteria in general exhibit significant metabolic redundancy and flexibility in synthesising EAAs (Cotton et al. 2020), but it is difficult to predict how this influences $\delta^{13}C$-EAA patterns. Based on biosynthesis pathways, phenylalanine emerges as the most likely candidate to cause consistent divergence in $\delta^{13}C$-EAA patterns between plants and algae, and bacteria and fungi. In terms of differential flow rates influencing $\delta^{13}C$-EAA patterns, there are two systematic differences between broad basal organism groups. The first is intracellular compartmentalisation within eukaryotes that affects the synthesis pathways of the five EAAs (Figure 1), potentially separating bacteria $\delta^{13}C$-EAA patterns. The second are differences in downstream demands for secondary metabolites. The synthesis and incorporation of lignin into vascular plant cell walls uses phenylalanine as a monomer precursor (Vanholme et al. 2010) and therefore may influence plant $\delta^{13}C$-EAA patterns. As lignin is relatively deplete in ^{13}C compared to other major biomolecules in plant tissues (Benner et al. 1987), its synthesis should result in an enriched ^{13}C pool of phenylalanine for proteins. In contrast, the cell walls of other major basal organism taxa do not require significant amounts of the five EAAs (Kottom et al. 2017, Domozych 2019, van Heijenoort 2001). Taken together, the unique biosynthesis pathways of phenylalanine, along with the distinct characteristics of intracellular compartmentalization and downstream demands for secondary metabolites, highlight the complexity of $\delta^{13}C$-EAA patterns across various life domains. Phenylalanine's divergent synthesis between plants, algae, bacteria, and fungi, combined with its utilisation in cell wall structures, makes it a central candidate in understanding and differentiating $\delta^{13}C$-EAA patterns within these groups.

To test the mechanistic expectation of phenylalanine, and explore other lineage specific $\delta^{13}C$-EAA patterns, we compiled and analysed data of 680 samples from 20 ecological and archaeological studies (Figure 4, Appendix S2). We applied linear discriminant analysis (LDA), which separates groups by maximising the differences between groups while minimising within-group variability, providing EAA specific weightings for group separation. To quantify the degree of group separation, we calculated pairwise Bhattacharya coefficients (BCs, Bhattacharyya 1946) on the LDA transformed data. BCs are a general measure of similarity between two multivariate distributions, with 0 indicating no overlap and 1 indicating identical distributions. We observe that plant $\delta^{13}C$-EAA patterns diverge from the other major basal resource groups, including algae (median overlap of 0.53, Figure 4a), due to increased phenylalanine $\delta^{13}C$ offsets. Bacterial $\delta^{13}C$-EAA patterns also separate from other basal resource groups, predominantly due to leucine (median overlap with plants of 0.23), while valine $\delta^{13}C$ offsets cause some divergence of fungi. Threonine $\delta^{13}C$ offsets contribute little to between basal resource group separation (Figure 4).

While algae δ^{13}C-EAA patterns express considerable variation, substructuring can be observed among the three clades of macroalgae. Brown (Phaeophyta) and red macroalgae (Rhodophyta) δ^{13}C-EAA patterns appear to separate (median overlap 0.35), but green macroalgae (Chlorophyta) occupy the overlapping δ^{13}C-EAA pattern space in between. Comparing macroalgae against seagrasses, the only marine vascular plants, shows that within the same biome, plant-algae separation is still driven by enriched phenylalanine δ^{13}C offsets. This is similarly the case when contrasting seagrasses with microalgae (Appendix S2: Figure S2b), where the δ^{13}C-EAA patterns of phytoplankton diverge between freshwater and marine biomes (median overlap of 0.33). Terrestrial plant δ^{13}C-EAA patterns do not discriminate on their C_3 or C_4 photosynthetic carbon fixation systems (median overlap of 0.91, Figure 4c). However, limited observations suggest separation for CAM plants, here solely represented by two cacti species from a single study (median overlaps of 0.32 and 0.25 with C_3 and C_4 plants respectively, Figure 4c). This is unexpected as CAM physiology affects fractionation during carbon acquisition, and therefore should only influence the baseline δ^{13}C-EAA values (section 2). Some individual C_3 plants express similar δ^{13}C-EAA patterns to CAM plants (Figure 4c). We explored substructuring of δ^{13}C-EAA patterns within terrestrial plants using multivariate Bayesian mixed models, as their phylogenetic diversity was well represented (212 samples across 18 families, Figure 4d, Appendix S3). Approximately half (36-66%) of the variation in δ^{13}C-EAA patterns in terrestrial plants can be attributed to phylogeny. The cacti CAM plants closely align with two other arid adapted C_3 plant families, Agavoideae and Zygophyllaceae, driven by increasing isoleucine but decreasing leucine δ^{13}C offsets (Figure 4d). Despite phenylalanine separating plants from other basal organism groups, phenylalanine along with valine contribute little to δ^{13}C-EAA pattern substructuring within plants. The contrast between valine and isoleucine is noteworthy as they have parallel synthesis pathways and therefore observed differences cannot be due to separate reactions or enzymes.

Taken together, we deduce that δ^{13}C-EAA patterns are predominantly driven by differences in flow rates of EAAs, particularly for substantial and continuous downstream demands as precursors for biopolymers. Despite sharing the same biosynthesis pathway, plants and algae separate based on phenylalanine δ^{13}C offsets, with plant phenylalanine being comparatively enriched in ^{13}C (Figure 4a), even among finer comparisons between sympatric plant and algal clades (Figure 4b, Appendix S2: figure S2b). Phenylalanine contributed little to δ^{13}C-EAA pattern variability within vascular plant phylogeny, which is consistent with the ubiquity of lignin synthesis in this group. The observation that δ^{13}C-EAA pattern distinctions can be observed not only with phylogeny, but also by ecosystems such as marine versus freshwater algae (Appendix S2: figure S2b) and similarities between arid climate adapted plants (Figure

4d), further highlights how consistent phenotypic expressions may contribute to δ^{13}C-EAA pattern variation. Several other studies have observed δ^{13}C-EAA pattern distinctions between organs of individual plants, i.e. roots, seeds, and leaves (Lynch et al. 2011, Larsen et al. 2016b, Jarman et al. 2017), demonstrating that structural differences can underpin δ^{13}C-EAA patterns within the same individual.

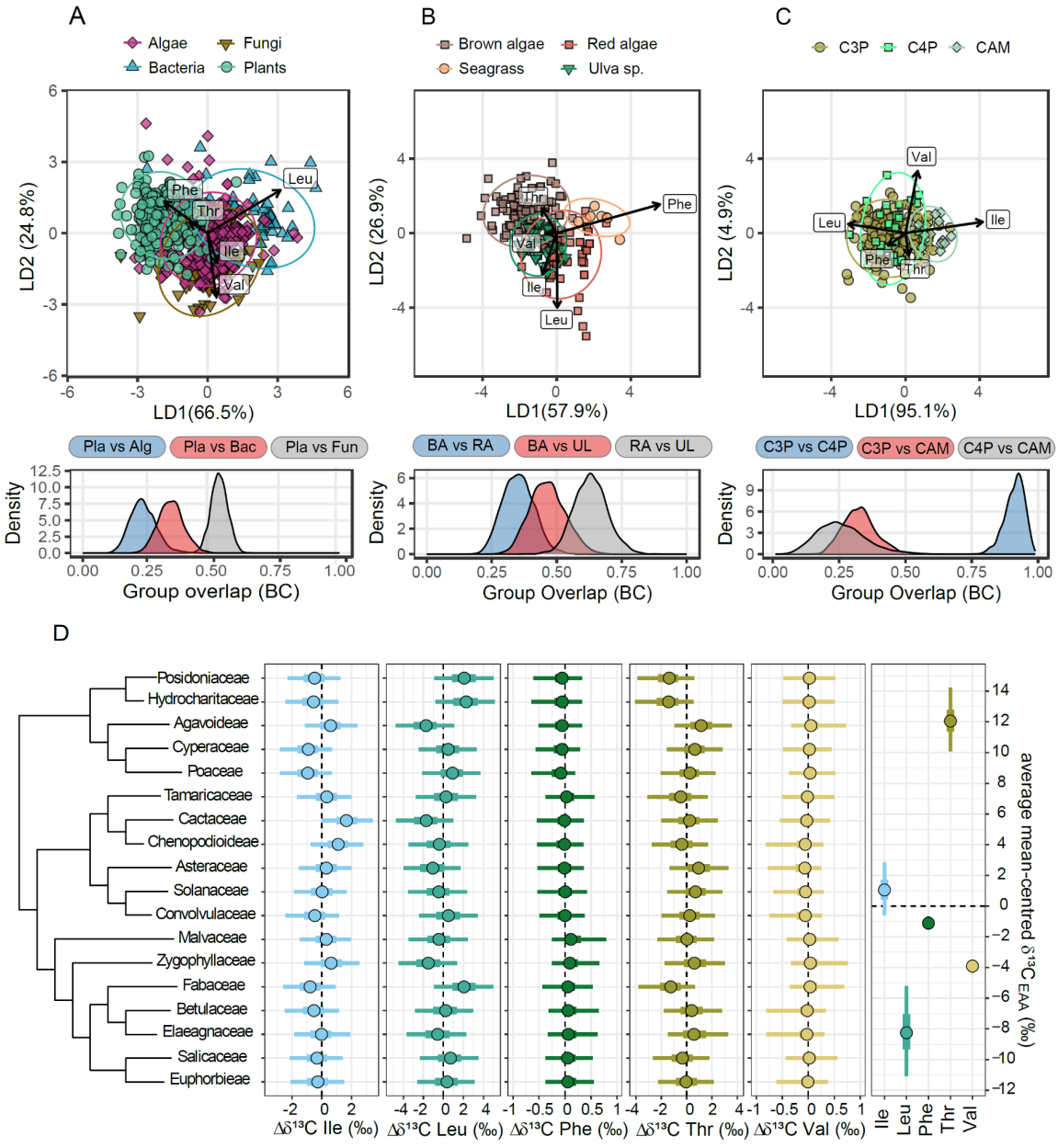

Figure 4. Linear discriminant (LD) analysis of mean-centred δ^{13}C-EAA values compiled from the literature (see Appendix S2). Upper subplot panel: LD scores for individual samples, with distinct symbols denoting each group. Lower subplot panel: posterior distributions of group pair overlaps, quantified by the Bhattacharyya coefficients (BC, see Appendix S2), indicating the probability density of degree of overlap in LD scores between groups (0 = no

overlap, 1 = identical distributions). EAAs considered: leucine (Leu), isoleucine (Ile), valine (Val), threonine (Thr), and phenylalanine (Phe). Each subplot features the following taxa: A) Heterotrophic bacteria, plants, and phytoplankton; B) Brown macroalgae, red macroalgae, green macroalgae (represented by *Ulva* sp.), and seagrasses; C) C_3 plants, C_4 plants, and CAM plants, containing the two cacti species *Cylindropuntia* sp. and *Opuntia* sp. For visual clarity, LD weighting coefficients for each EAA were multiplied by 8. D) Modelled mean-centred δ^{13}C-EAA values (δ^{13}C-EAA patterns) of vascular plants, showing the global average values (right hand panel) and individual EAA offsets, $\Delta\delta^{13}$C among the 18 taxonomic (sub)families in the vascular plant dataset. Phylogenetic topology is plotted on the left hand side. Circles indicate median posterior values, thick bars denote the 50% credible intervals (CIs) and thin bars the 95% CIs. Average mean-centred δ^{13}C CIs for phenylalanine and valine fall within the median circles.

3.2. Considerations for the δ^{13}C-EAA patterns of facultative prototrophs

Until now, we presumed that the EAAs that define a basal organism's δ^{13}C-EAA pattern are exclusively the result of de novo synthesis. This is true for strictly autotrophic organisms, as they are obligate EAA-prototrophs. However, basal organisms with the capacity to take up external sources of organic carbon, i.e. heterotrophy, have the potential to directly assimilate external AAs into their tissues, termed facultative AA-prototrophy. This is not only limited to heterotrophic bacteria and fungi, but also includes mixotrophic basal organisms that can both fix inorganic carbon and acquire external organic carbon. Many algal protists, free-living protozoa and greens plants may be classed as mixotrophs (Matantseva and Skarlato 2013, Selosse and Roy 2009). If facultative EAA-prototrophs incorporate substantial amounts of externally derived EAAs, then their in situ δ^{13}C-EAA patterns will not wholly reflect the carbon fractionation among EAAs of their de novo synthesis. This affects the accuracy of δ^{13}C-EAA pattern applications tracing the origin of basal resources (Arsenault et al. 2022a), and therefore requires knowledge on the occurrence, degree, and flexibility of facultative prototrophy in different basal resource groups.

Assimilating AAs opportunistically from the external environment is energetically efficient compared to synthesising them de novo (Morrissey et al. 2023), however basal organisms must have the necessary membrane proteins that are energetically expensive to synthesise and maintain. The capacity for AA assimilation across heterotrophic bacteria is common but phylogenetically constrained, implying genetic and phenotypic prerequisites for AA membrane transport proteins (Dang et al. 2022). The energetic cost of AA biosynthesis is a considerable evolutionary selection pressure for bacteria (Heizer et al. 2006, D'Souza et al. 2014), suggesting that demand for external AAs will be substantial and highly competitive. Functional specialisation within soil microbial communities is apparent (Morrissey et al. 2023), with some bacteria being auxotrophic (Table 1), having lost their biosynthesis capacity for certain AAs and becoming metabolically dependent on external AA sources (Heizer et al. 2006, D'Souza et al. 2014).

Conversely, saprotrophic bacteria that undertake biochemical decomposition of complex polymers do not assimilate appreciable amounts of simple organic carbon compounds including AAs (Dang et al. 2021). Various AA transport proteins occur in fungi (Bianchi et al. 2019), however saprotrophic fungi are likewise specialised in breaking down and assimilating large insoluble polymers through exoenzyme secretion (Algora Gallardo et al. 2021, Batista García et al. 2016, Ruiz-Dueñas et al. 2021). This contrasts with root-associated mycorrhizal fungi that rely on simple carbon compounds from plants, but observed two-way carbon exchanges imply mixotrophy may occur in fungi-hosting vascular plants (Bolin et al. 2017, Selosse et al. 2016, Firmin et al. 2022), beyond the limited cases of carnivory and hemi-parasitism (Giesemann and Gebauer 2022, Selosse and Roy 2009). AA membrane transport proteins have been characterised in several species of plant roots, the prerequisite for direct uptake of external AAs (Näsholm et al. 2009, Moe 2013). Although mixotrophic protists that phagocytose prey have long been recognised (Jones 2000, Sanders 1991), uptake of external carbon in the form of simple dissolved compounds, including AAs, has also been observed to occur in more traditionally viewed autotrophic microalgal species such as diatoms (e.g. Rivkin and Putt 1987, Tuchman et al. 2006). The potential for uptake of external biomolecules in these algae and plants likely evolved as an adaptation to nutrient rich but light-limited, and therefore carbon limited, environments (Selosse et al. 2017).

Examples of facultative prototrophy may therefore be found across basal organism groups. However, the uptake of external AAs alone may not result in significant AA assimilation into tissues if the external AAs are preferentially used for other metabolic purposes, or only occurs under certain physiological conditions. In diatoms, external AA uptake has been observed when cultivated under dark conditions but occurs with simultaneous increases in oxidation rates, implying external AAs are used to fuel respiration (Tuchman et al. 2006). Antarctic diatoms can incorporate the carbon of external AAs into their proteins (Rivkin and Putt 1987), suggesting AA uptake in algae occurs as a physiological response to carbon limitation when photosynthesis is restricted due to prolonged dark periods. Culturing fungi under very high AA concentrations led to changes in δ^{13}C-AA patterns, implying incorporation of external AAs (Arsenault et al. 2022a). Labelling experiments demonstrate however that uptake of external AAs occurs during exponential but not stationary growth phases in fungi (Martin-Perez and Villén 2015). For bacteria, specialised adaptations suggest that external AAs will benefit only those species that readily utilise labile dissolved organic carbon (Morrissey et al. 2023, Dang et al. 2022). Dissolved AAs in soils and aquatic environments typically occur in low concentrations of 0.01-50 µM and 1-10 µM respectively (Lytle and Perdue 1981, Kielland 1994, cf. 130-840 µM in “low” AA concentration treatment in Arsenault

et al. 2022a). This suggests that under most conditions facultative prototrophs do not assimilate enough external EAAs into their tissues to substantially alter their δ^{13}C-EAA patterns.

While some specific environments may induce high external AA uptake in some basal organisms, the evidence suggests that this is not a common occurrence. Reflecting on our compiled δ^{13}C-EAA data, we can exclude the influence of external EAAs influencing δ^{13}C-EAA patterns de facto as bacteria, fungi and microalgae are predominantly cultured in AA-free media. This ensures that measured EAAs are derived from the organisms' biosynthetic pathways and not from the culturing substrate (Larsen et al. 2009), and therefore can be applied for determining basal resource origin. It should be acknowledged that culture media cannot mimic the complex natural growth environments experienced in situ, especially for saprotrophic organisms. However, carefully designed cultivation experiments comparing δ^{13}C-EAA patterns between EAA-free and isotopically labelled EAA media under natural growth conditions could provide insights into the metabolic dependencies of facultative EAA-prototrophs on external EAAs.

3.3. From δ^{13}C-EAA patterns to fingerprints

The variety of phylogenetic and ecological factors that influence δ^{13}C-EAA patterns prompts the question how to define the δ^{13}C-EAA fingerprint for a given basal resource. The concept of a "fingerprint" for δ^{13}C-EAA patterns, as introduced by Larsen et al. (2009) to differentiate between bacterial, fungal, and plant EAA biosynthesis, has since been applied to a wider range of contexts (e.g. Larsen et al. 2012, Arthur et al. 2014, Yun et al. 2022). The notable lack of a formal definition of a δ^{13}C-EAA fingerprint likely contributed to variations in the construction and interpretation of "δ^{13}C-EAA fingerprints", such as the use of measured rather than mean-centred δ^{13}C-EAA values (e.g. Besser et al. 2022), or referring to consumer δ^{13}C-EAA patterns as "fingerprints" (e.g. McMahon and Newsome 2019). Reflecting on the original purpose of δ^{13}C-EAA fingerprints, which was to trace the contribution of different basal resources to consumer tissue proteins (Larsen et al. 2009), we explicitly define a "δ^{13}C-EAA fingerprint" as:

"*the minimum δ^{13}C-EAA pattern space that is solely occupied by a group or collection of similar basal organisms and encompasses the intragroup variability in δ^{13}C-EAA patterns expressed by those organisms.*"

Here, the 'uniqueness' characteristic of a δ^{13}C-EAA fingerprint is qualified by sole occupancy of a basal organism group in δ^{13}C-EAA pattern space. By limiting it to the minimum occupied space, arbitrary overlaps between basal resources can be excluded. However, the sole occupancy of δ^{13}C-EAA pattern space is comparative, and therefore depends on the presence or absence of other basal organisms in an ecosystem (shown in Figure 3c, basal resource groups 3 & 4) or its relevance to the consumer (section 4.1). A priori understanding of a consumer's ecology and its ecosystem underpins which basal resource δ^{13}C-EAA patterns will be defined as δ^{13}C-EAA fingerprints. Therefore, δ^{13}C-EAA fingerprints will be study and context specific, and may change between studies that include the same basal organisms.

To define groups of similar basal organisms, a flexible framework is needed to accommodate the variety of studies using δ^{13}C-EAA fingerprints. Phylogenetically closer organisms are expected to express more similar δ^{13}C-EAA patterns due to genetic constraints associated with AA biosynthesis, as we observed in broad basal resource groups (Figure 4a,b). Yet, adaptations to particular environments can lead to similar δ^{13}C-EAA patterns among phylogenetically distant groups, such as arid adapted plants (Figure 4d). Variation in δ^{13}C-EAA patterns occurs across varying levels of phylogeny, and can be driven by different EAAs (section 3.1, Figure 3a,b and 4d, Appendix S3: figure S3a). These observations suggest that δ^{13}C-EAA patterns have the potential to express higher specificity than is acknowledged in the literature, where broad basal resource groups are characterised (Arthur et al. 2014, Ayayee et al. 2015, McMahon et al. 2015a, Rowe et al. 2019, Macartney et al. 2020, Wall et al. 2021, Arsenault et al. 2022b, Stubbs et al. 2022). Valuable phenomenological insights have been provided over the past decade, however, we propose the development of a framework focused on the metabolic functioning of basal organisms (section 2) to facilitate predictions of δ^{13}C-EAA pattern distinctions across clades and environments to complement the current in situ measurements on a study by study basis.

3.4. Optimal characterisation of δ^{13}C-EAA fingerprints

Defining δ^{13}C-EAA fingerprints requires a conscientious approach in basal organism sampling and analysis. The characterisation of δ^{13}C-EAA fingerprints involves the accurate representation of a particular basal organism group in an ecosystem, its natural variation, and its relevance to the studied consumer. For optimally characterising δ^{13}C-EAA fingerprints, the following considerations are important:

- Basal organism samples should accurately represent the taxonomic group under investigation in the studied ecosystem. This precludes composite samples such as particulate organic matter

filtrates, microalgal and bacterial mats, or partially degraded materials (detritus) that are contaminated with faeces, degraded organic matter, bacteria etc. Further, composites average over a diversity of species, preventing specific characterisation.

- Tissue samples of specialist primary consumers (e.g. zooplankton or herbivorous fishes) are often used as a surrogate for specific basal resource δ^{13}C-EAA fingerprints (e.g. Skinner et al. 2021). However, sole dependency of a primary consumer on one specific basal resource is unlikely due to incidental EAA assimilation from other sources (e.g. functionally similar basal resources, detritus, associated bacteria and meiofauna in macroalgal turfs, Nicholson and Clements 2023).
- Prior to in situ sampling, systematic characterisation of δ^{13}C-EAA fingerprints in singularly cultured basal organisms would establish the extent to which basal resources can be subdivided into clades with similar δ^{13}C-EAA patterns. Field collected samples with a high concentration of a particular species or clade can be analysed for verification, as some basal resources might display different δ^{13}C-EAA patterns in situ compared to cultures. For example, δ^{13}C-EAA patterns of the sub-ice algae *Melosira arctica* growing in long-chained strands in its natural under-ice habitat significantly differed from their cultivated form of singular cells (Vane et al. 2023).
- The extent to which unique δ^{13}C-EAA fingerprints can be characterised depends on the number of EAAs measured in basal organisms and metazoan tissues due to analytical limitations (section 6.1 and 7). In most proteinaceous soft tissues 6-7 EAAs can be measured, but is reduced in mineralised tissues such as biogenic calcites due to lower EAA concentrations (McMahon et al. 2018, Vokhshoori et al. 2022). It is advisable to reliably measure as many EAAs as possible (section 7) to increase the discriminatory power of δ^{13}C-EAA patterns.

Directly visualising whether $\delta^{13}C_{EAA}$ patterns of select basal organism groups solely occupy their isotopic space, and therefore are a δ^{13}C-EAA fingerprint (section 3.3, Figure 3c), is not feasible due to the high dimensionality of the data. Visualising multiple pairwise biplots of mean-centred δ^{13}C-EAA values results in significant information loss and can be difficult to interpret. Dimension reduction approaches used to visualise δ^{13}C-EAA patterns include principal component analysis (PCA, which maximises total variation across the dataset) and LDAs (section 3.1). While LDAs may seem more appropriate to identify distinctions between δ^{13}C-EAA patterns as it aims to maximise group separation, PCA can outperform LDA in separating groups when sample sizes are small (Martínez et al. 2001; for a comparison of the two approaches see Appendix S4). The distinctions between δ^{13}C-EAA patterns can be objectively quantified with e.g. Bhattacharya coefficients (Bhattacharyya 1946, see section 3.1, Figure 4a,b,c, Appendix S2).

Quantifying δ^{13}C-EAA pattern distinctions not only improves statistical clarity for defining δ^{13}C-EAA fingerprints, but facilitates more direct comparisons between studies that measure different suites of EAAs.

4. Tracing basal resources from a consumer perspective

Organisms consume the basal resources synthesised by basal organisms either directly or indirectly through their prey. In doing so, they assimilate the baseline δ^{13}C-EAA values, and by extension the δ^{13}C-EAA patterns, of those basal resources into their own tissues with minimal alteration. Consumers' δ^{13}C-EAA patterns are a weighted average of the assimilated δ^{13}C-EAA patterns that can be used to identify the basal organisms that synthesised the basal resources using δ^{13}C-EAA fingerprints. Prior knowledge of the consumer's dietary niche is essential to characterise relevant basal resources, and to determine the extent of the distinction and specificity with which basal resources should be quantified. While δ^{13}C-EAA fingerprints can trace basal resources to broad taxonomic groups and specific clades of basal organisms, their effectiveness depends on the research question and inferences become more nuanced for consumers that partially rely on EAAs biosynthesized by (endo)symbionts. In this section, we discuss how δ^{13}C-EAA patterns and fingerprints can be applied to robustly infer basal resource use by consumers.

4.1. Applying δ^{13}C-EAA fingerprints in ecological studies

δ^{13}C-EAA fingerprints can differentiate basal resources across broad taxonomic groups and finer clades (section 3). However, disentangling these from δ^{13}C-EAA patterns of consumer tissues is challenging, especially for higher trophic level consumers that acquire basal resources through multiple trophic transfers. A first step is to determine to which level basal resources should be distinguished. General questions might involve differentiating between aquatic versus terrestrial basal resources (Larsen et al. 2013, Liew et al. 2019). More complex inquiries can revolve around estimating the proportional use of multiple basal resources by a consumer, or distinguishing among closely related clades such as phytoplankton groups (McMahon et al. 2015a, Vane et al. 2023). While characterising basal organisms to clades is potentially powerful, researchers need to consider when such fine-scale distinctions no longer inform about the ecological processes of interest. For example, distinguishing between various clades within phytoplankton will not be informative when phytoplankton make only a minor contribution to consumer biomass (Vane et al. 2023). After thoroughly characterising δ^{13}C-EAA fingerprints within the

research framework, their variation has to be evaluated together with the consumer tissue δ^{13}C-EAA patterns using methods such as biplots and, or PCA/LDAs. If consumer δ^{13}C-EAA patterns fall outside of known δ^{13}C-EAA fingerprints, this can indicate an unaccounted basal resource or incomplete characterisation due to limited replication or sampling. Biases during isotopic analysis can also lead to offsets between consumer and basal resources (section 7). These considerations are important for reliable quantifications of proportional basal resource use by the consumer (section 8).

Many researchers rely on existing training data sets, i.e. basal resource δ^{13}C-EAA values characterised in other studies, such as those of Larsen et al. (2013) and McMahon et al. (2016), to infer basal resource use. Generic training datasets assume that basal resource δ^{13}C-EAA patterns are highly conservative with broad ecosystem applicability, a questionable assumption at broad taxonomic scales. As elaborated in section 3, variations within δ^{13}C-EAA patterns of broad taxa such as microalgae and bacteria may be attributed to finer phylogenetic substructuring or associated with phenotypic structural traits. Within individual plants, δ^{13}C-EAA fingerprints vary among seeds, roots, and leaves (Lynch et al. 2011, Larsen et al. 2016b, Jarman et al. 2017), necessitating sampling of specific plant organs that are ingested by the consumer. Using generic training data therefore introduces variation that is not pertinent to the specific ecosystem, undermining discrimination between basal resources and distorting the true underlying δ^{13}C-EAA pattern space that comprises the consumer tissue (Liew et al. 2019, Macartney et al. 2020, Phillips et al. 2020, Stubbs et al. 2022). Moreover, without proper inter-laboratory calibration, training data may contain inconsistencies arising from different analytical protocols and errors that are currently not well-constrained and therefore difficult to account for with calibrations post hoc (section 7). While researchers often enrich training data with ecosystem-specific δ^{13}C-EAA measurements (e.g. Arthur et al. 2014, Ayayee et al. 2015, Rowe et al. 2019, Macartney et al. 2020, Wall et al. 2021, Arsenault et al. 2022b, Stubbs et al. 2022), for accurate inferences it is advisable to create study-specific δ^{13}C-EAA fingerprints of relevant basal resources. Moving forward, a δ^{13}C-EAA fingerprint library could streamline this process, if built on widely accepted international reference materials and standardised methodologies (section 7). Such a library would be invaluable in addressing large-scale ecological questions over various spatiotemporal scales.

4.2. Consumers with (endo)symbionts

The direct assimilation of basal resources by consumers can be compounded by the occurrence of symbiotic relationships. Endosymbionts often supplement hosts with de novo synthesised EAAs,

particularly when the host specialises on nutrient-poor diets. Examples include aphids and other plant sap feeding insects with sugar dominated diets (Akman Gündüz and Douglas 2009), or detrital consumers, such as earthworms, springtails, and termites (Ayayee et al. 2015, Larsen et al. 2016a). EAA supplementation can vary dynamically depending on dietary availability and digestibility, leading to trade-offs. For instance, experimentally increasing indigestible fibre content in enchytraeids' diets increased EAA supplementation by gut symbionts, but reduced enchytraeid growth (Larsen et al. 2016a).

In marine environments, mixotrophic holobionts such as corals, molluscs, and sponges rely on a complex community of symbionts in addition to heterotrophic feeding (Skinner et al. 2022, Pita et al. 2018). These include dinoflagellate algae hosted within coral tissues (Skinner et al. 2022); diverse endolithic microbiomes associated with the carbonate skeleton, including microalgae, fungi, and bacteria (Pernice et al. 2020); and epidermal and gastrodermal mucus microbiomes (Fox et al. 2019, Kwong et al. 2019). Coupled host-endosymbiont δ^{13}C-AA values suggest that endosymbiotic algae play a major role in the biosynthesis and provisioning of AAs, but transfer of photoassimilates also occurs between endolithic symbionts and overlying host tissues (Schlichter et al. 1995, Fine and Loya 2002). Yet the biochemical roles of holobiont symbioses extend beyond AA provisioning, including rapid carbon fixation and subsequent high-energy biomolecule transfers (Kopp et al. 2015, Tremblay et al. 2012).

Identifying and quantifying EAA contributions to host consumer tissues by symbionts requires the characterisation of δ^{13}C-EAA fingerprints of both symbionts and host diet. δ^{13}C-EAA patterns in dinoflagellate endosymbionts of corals can be distinct from the surrounding particulate organic matter, a proxy for phytoplankton (Fox et al. 2019, Wall et al. 2021) and other free living dinoflagellates (Stahl et al. 2023). However, similar characterisations are largely missing for other symbionts like those of sponges (Shih et al. 2020). For microbial gut symbionts, their δ^{13}C-EAA patterns remain to be adequately characterised, despite the possibility to cultivate gut microbes from model organisms such as *Drosophila* (Erkosar et al. 2013). Currently, researchers predominantly rely on training data from disparate terrestrial bacteria to identify gut microbial EAA supplementation (e.g. Arthur et al. 2014), which may yield inaccurate quantifications (see section 4.1). Although extensive research is required to capture the full variation and distinction of δ^{13}C-EAA patterns among different symbiont taxa, an alternative approach involves estimating these δ^{13}C-EAA patterns from the offsets between δ^{13}C-EAA values of diet and consumer tissues (Larsen et al. 2016b, Newsome et al. 2020). However, this method requires prior knowledge of the proportional contributions of each EAA from symbionts to consumer tissues which are generally poorly constrained. Future studies should also consider the spatiotemporal host-symbiont

dynamics when attempting to acquire relevant symbiont δ^{13}C-EAA fingerprints. As symbionts are typically hosted in diverse communities, the optimal characterisation of symbiont δ^{13}C-EAA patterns will likely be difficult beyond isolating single symbiont species cultures. Nonetheless, with symbiont δ^{13}C-EAA fingerprints characterised, they could aid in identifying and quantifying changes in EAA symbiont provisioning to their host.

5. Beyond δ^{13}C-EAA fingerprints

Consumers may rely on a variety of basal resources that do not possess distinct δ^{13}C-EAA patterns and therefore lack δ^{13}C-EAA fingerprints. For example, researchers might aim to differentiate contributions from phylogenetically similar understory vs canopy vegetation, or sea-ice microalgae vs pelagic phytoplankton (de la Vega et al. 2019, Tejada et al. 2020). Spatial or environmental segregation of these basal resources within the ecosystem (e.g. ice-algae vs. phytoplankton), or differences in carbon fixation machinery (e.g. C3 vs. C4 plants) can result in disparate bulk δ^{13}C baseline values between basal organism groups, despite their δ^{13}C-EAA patterns being similar. As measured δ^{13}C-AA values combine the basal organism AA specific biosynthesis offsets with potentially disparate baselines (section 2.1, Figure 1), opting for measured δ^{13}C-AA data rather than mean-centred δ^{13}C-EAA values in consumers can aid in differentiating food web contributions in such instances. By applying multivariate analyses to measured δ^{13}C-EAA data, researchers have distinguished between freshwater algae, marine algae, terrestrial matter, and detrital material simultaneously in consumers (McMahon et al. 2016, Vane et al. 2018; 2023, Johnson et al. 2019). Incorporating δ^{13}C-NEAA values could provide further insight into macronutrient sources and the physiological conditions of animals (Barreto-Curiel et al. 2017). However, drawing such inferences from δ^{13}C-NEAA values remains challenging and underexplored (Larsen et al. 2022aa) as the extent to which individual NEAAs reflect metabolic versus dietary sourcing is not yet fully understood. Here, we provide an overview for integrating full δ^{13}C-AA datasets into ecological studies. We discuss the factors that influence δ^{13}C-NEAA values in animals by expanding our conceptualisations from section 2, explore the utility of PCA and LDA for δ^{13}C-AA data analysis, and examine whether individual NEAAs primarily reflect metabolic or dietary influences.

5.1. Factors affecting δ^{13}C-NEAA values in animals

While EAAs in consumer tissues must be directly routed from the diet, the NEAAs are a mixture of two sources: NEAAs that are synthesised de novo by the organism and those assimilated from the diet (ca. Figure 1 to Figure 5). Carbon for NEAA synthesis comes from various macronutrients, each with its own unique isotopic composition, associated catabolic pathways, and contribution to NEAA biosynthesis (see Figure 5 and Appendix S1: Figure S1C for a detailed metabolic network). For instance, lipids and short-chain fatty acids are generally depleted in ^{13}C compared to proteins and carbohydrates (Deniro and Epstein 1977, Melzer and Schmidt 1987, Weber et al. 1997). NEAAs directly assimilated from the diet will have δ^{13}C values mirroring those of the dietary sources; however, they may undergo substantial metabolic processing, particularly in the splanchnic tissues (e.g., liver, stomach, intestines, etc.), that could result in isotope fractionations (Caut et al. 2009, Larsen et al. 2022a). Additionally, dietary sourced AAs may experience fractionation during their catabolic processing within the microbiome of the abdominal cavity. The body's response to changes in diet quality may vary depending on the specific AAs involved, as different macronutrients enter varying segments of the central metabolic network (Appendix S1: Figure S1C). For example, glycine metabolism responds to dietary AA composition whereas alanine metabolism responds to energy balance and carbohydrate intake rather than to dietary AA content (Yu et al. 1985).

NEAA deficiency or general caloric restrictions can prompt heightened catabolism (and splanchnic retention) of certain AAs, making them unavailable for the formation of structural tissues (Neis et al. 2015). In humans, retention rates of dietary EAAs within the abdominal cavity are low, ranging from 20% to 50% with the exception of threonine, which has a 90% retention rate. Retention rates for dietary NEAAs tend to be higher, but variable. Differentiating these retention rates between digestive processes and tissue protein synthesis is complex (Battezzati et al. 1999, Dai et al. 2012). Once dietary NEAAs reach the liver - the centre of AA degradation and synthesis - they serve various functions, including as building blocks for proteins and precursors for non-proteinogenic metabolites (Burrin and Stoll 2009, Figure 5). Excess dietary NEAAs are converted into fat, which can later be catabolized into glycogen as needed. Although the precise ratio of dietary to synthesised NEAAs in proteinogenic tissues often remains ambiguous due to fluctuating metabolic demands and catabolic rates, it is feasible to make reasonably accurate estimates in certain tissues like collagen when considering NEAAs as an aggregated pool (Hobbie et al. 2017).

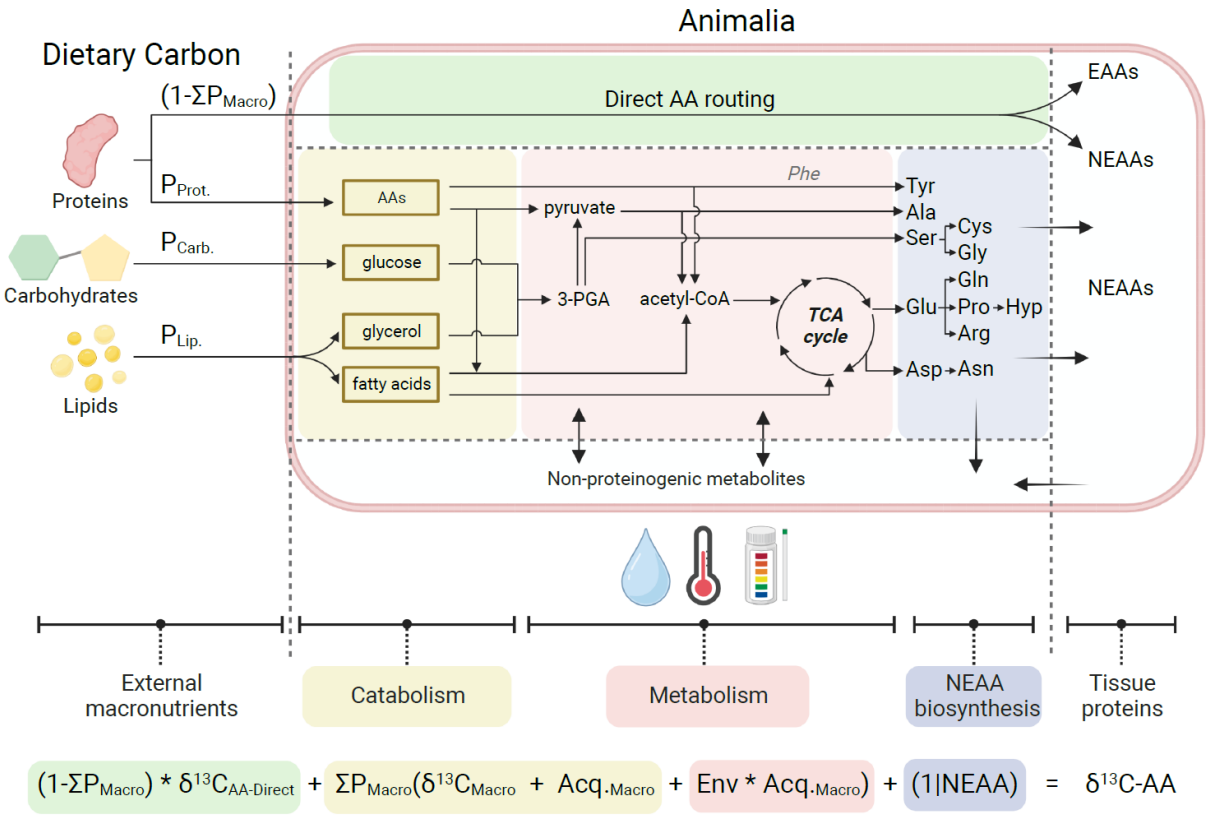

Figure 5. Conceptual schematic incorporating macronutrients (proteins, carbohydrates, lipids), metabolic processes, and environmental effects that contribute to the δ^{13}C-AA values in animals (ca. Figure 1). Metabolic processes are divided into macronutrient catabolism, central metabolism including glycolysis and the tricarboxylic acid (TCA) cycle, and the biosynthesis of the non-essential AAs (NEAAs) that can be utilised for proteinogenic or non-proteinogenic purposes. All the essential AAs (EAAs) are assumed to be routed directly from dietary proteins ($\Sigma P_{Macro} = 0$). A fraction of the dietary NEAAs may be routed directly to tissue proteins ($1 - \Sigma P_{Macro}$), which will have δ^{13}C values that reflect those of the dietary NEAAs. In terms of the sources and processes affecting δ^{13}C-NEAA values of tissue proteins, the molecular constituents of each macronutrient have their own initial isotopic composition, $\delta^{13}C_{Macro}$, and fractionation, $Acq._{Macro}$, as they are converted to NEAA-precursors. As the catabolic networks are different for the three macronutrients (Appendix S1: figure S1C), the effect of environment will likely induce different physiological responses in isotopic fractionations ($Env*Acq._{Macro}$). The contributions of different macronutrients to NEAA synthesis ($\Sigma P_{Macro} = P_{Prot.} + P_{Carb.} + P_{Lip.}$) may fluctuate with diet composition and covary with physiological changes such as the accumulation of adipose tissue, reproduction status or muscle catabolism. Tissue proteins may be catabolised and re-enter the central metabolism. The metabolic pathways are summarised based on Stryer et al. (2019, see Appendix S1: figure S1C for a detailed metabolic network). Abbreviations: 3-PGA, 3-Phosphoglyceric acid; Ala, alanine; Arg, arginine; Asn, asparagine; Asp, Asparagine; Cys, cysteine; Gly, glycine; Gln, glutamine; Glu, glutamic acid; Hyp, hydroxyglycine; Pro, proline; Ser, serine; Tyr, tyrosine. The illustration was created with BioRender.com.

5.2. Exploring full $\delta^{13}C$ amino acid datasets

Many factors encompassing diet, digestive physiology, metabolism, and life history traits influence $\delta^{13}C$-NEAA values in consumers. Unravelling these complex interactions necessitates a comprehensive approach, especially when trying to distinguish metabolic from dietary effects. To isolate the factors contributing to $\delta^{13}C$-NEAA variations, examining data from closely related consumer species or functional groups can be helpful. This allows for establishing informed assumptions based on shared characteristicsamong consumers like digestive physiology, metabolism, and life histories (Larsen et al. 2022aa). Intriguingly, the most consistent and robust insights into $\delta^{13}C$-NEAA data have emerged from human studies when interpreted in concert with measured $\delta^{13}C$-EAA values and $\delta^{13}C$-EAA patterns (Corr et al. 2005, Choy et al. 2013, Yun et al. 2018, 2020, Johnson et al. 2021). Epidemiological studies have shown that $\delta^{13}C$-NEAA values in humans can vary with specific food intakes, but have so far explored only a limited spectrum of human diets (Choy et al. 2013, Yun et al. 2018, 2020, Johnson et al. 2021). This suggests that $\delta^{13}C$-NEAA variations can deepen our understanding of the complex interplay between consumer biology, and their diverse dietary sources.

To broaden our perspective on integrating $\delta^{13}C$-NEAA values for understanding resource use, we assembled archaeological $\delta^{13}C$-AA data from human bone collagen and hair keratin samples, covering a period of 6,500 years from diverse geographical locations (eight studies, n = 52; see Appendix S4 for details). This dataset includes $\delta^{13}C$ values for five NEAAs (alanine, aspartate/asparagine, glutamate/glutamine, glycine, proline) and two EAAs (phenylalanine, valine). From contextual archaeological information, we can presume that these AAs are derived from four major protein sources: freshwater (FP), marine (MP), terrestrial C_3 plants (C3P), and terrestrial C_4 plants (C4P). For a subset of the populations, there is sufficiently detailed archaeological data to make prior assumptions about the major protein sources in their diets. Individuals from this subset are denoted as ‘known’ individuals. For a comprehensive discussion on predictive accuracy with different ordination and preprocessing combinations within the data, see Appendix S4.

To explore how the relative offsets in AAs vary among individuals and populations, we employed PCA on EAA mean-centred $\delta^{13}C$-AA data (Figure 6-A1 and A2), with results showing relatively strong separation among the four protein sources. Most AAs align with PC1, which differentiates populations along a terrestrial (C3P and C4P) - aquatic (FP and MP) axis, while PC2 distinguishes between C3P/C4P and is primarily driven by the $\delta^{13}C$ contrast between proline and aspartate. These distinctions are further

pronounced by combining measured $\delta^{13}C$ values - data that comprises the individual $\delta^{13}C$-AA offsets combined with the $\delta^{13}C$ bulk baselines - with LDA that maximises group separation whilst minimising intra-group variation. The LDA highlights significant variability in the contribution of different AAs to group separation within measured $\delta^{13}C$ values. Phenylalanine and valine again distinguish between terrestrial and aquatic resources (Honch et al. 2012, Larsen et al. 2013), while proline and glutamate separates the C3P group. Glycine plays a key role in differentiating FP from other groups (Corr et al. 2005) whereas alanine and aspartate contributed very little to group separation. Interestingly, Fry et al. (2023) identified that both alanine and aspartate position specific ^{13}C carboxyl trends are strongly associated with lipid metabolism across a broad range of animals, including mammals, mollusks, fish, and crustaceans. This suggests that alanine and aspartate $\delta^{13}C$ values are more reflective of an individual's metabolic state than of their macronutrient sourcing. Conversely, proline appears to be the most source diagnostic of the NEAAs, which aligns with the fact that proline has one of the lowest splanchnic retention rates of NEAAs (~40%). Thus, our meta-analysis of humans suggests that a combination of metabolic effects in case of asparagine (Figure 6-A1 and A2) and source effects in case of proline, can separate C3P and C4P sources when $\delta^{13}C$-EAA patterns cannot (Figure 4C). To delve deeper into the multifaceted factors that drive variability in $\delta^{13}C$-NEAA values, we propose an investigative tandem of expanded meta-analyses coupled with detailed compound-specific and position-specific isotope analysis. This comprehensive approach has the potential to dissect the layers of complexity and identify the precise processes, metabolic activities or the influence of dietary sources, that are responsible for the variability observed in $\delta^{13}C$-NEAA values in consumers.

The efficacy of using measured rather than mean-centred $\delta^{13}C$-EAA values in multivariate analyses is closely tied to protein sources stemming from broad biomes - here terrestrial and aquatic. These biomes can be further divided into types of vegetation (C_3 vs. C_4) or water bodies (freshwater vs. marine), each having distinct baseline $\delta^{13}C$ values. Incorporating measured $\delta^{13}C$ values alongside source-specific $\delta^{13}C$-AA patterns in multivariate analyses compensates for the other's limitations while maintaining their strengths. This synergy is most effective when the intergroup variability, the differences in baseline $\delta^{13}C$ values between biomes, is markedly greater than the intragroup variability, or the variation within a single biome. Case in point is the meta-analysis above involving archaeological human samples, along with ecological studies by e.g. Vane et al. (2018) and Johnson et al. (2019). These studies all satisfy the prerequisite conditions for variability, enabling them to successfully differentiate between AA or protein sources that may have otherwise presented indistinguishable $\delta^{13}C$-EAA fingerprints. While multivariate

integration of measured δ^{13}C-AA values has proven fruitful under certain conditions, it is important to consider the limitations of this approach. Baseline δ^{13}C-AA values are sensitive to environmental fluctuations, making them context-dependent (McMahon et al. 2016, Vane et al. 2023). In situations where baseline δ^{13}C-AA values show subtle distinctions, a comprehensive sampling strategy becomes crucial. Seasonal or spatial sampling can help constrain the relevant mixing areas for each protein source, providing a more stable context for analysis (section 8.3, Vane et al. 2023). This allows for a more reliable estimation of baseline δ^{13}C values in environments where protein sources within a biome are not sharply delineated. While rigorous sampling can constrain subtle baseline δ^{13}C differences, this level of detail is hard to achieve when analysing historical or archaeological samples. These sources often present gaps in spatiotemporal data, complicating the construction of a robust analytical framework. Constraints such as limited sample sizes, material degradation, or incomplete records further limit our ability to achieve the ideal thoroughness achievable in contemporary studies. In these challenging scenarios, auxiliary data like climatic records or historical documentation may provide complementary information for constraining protein sources.

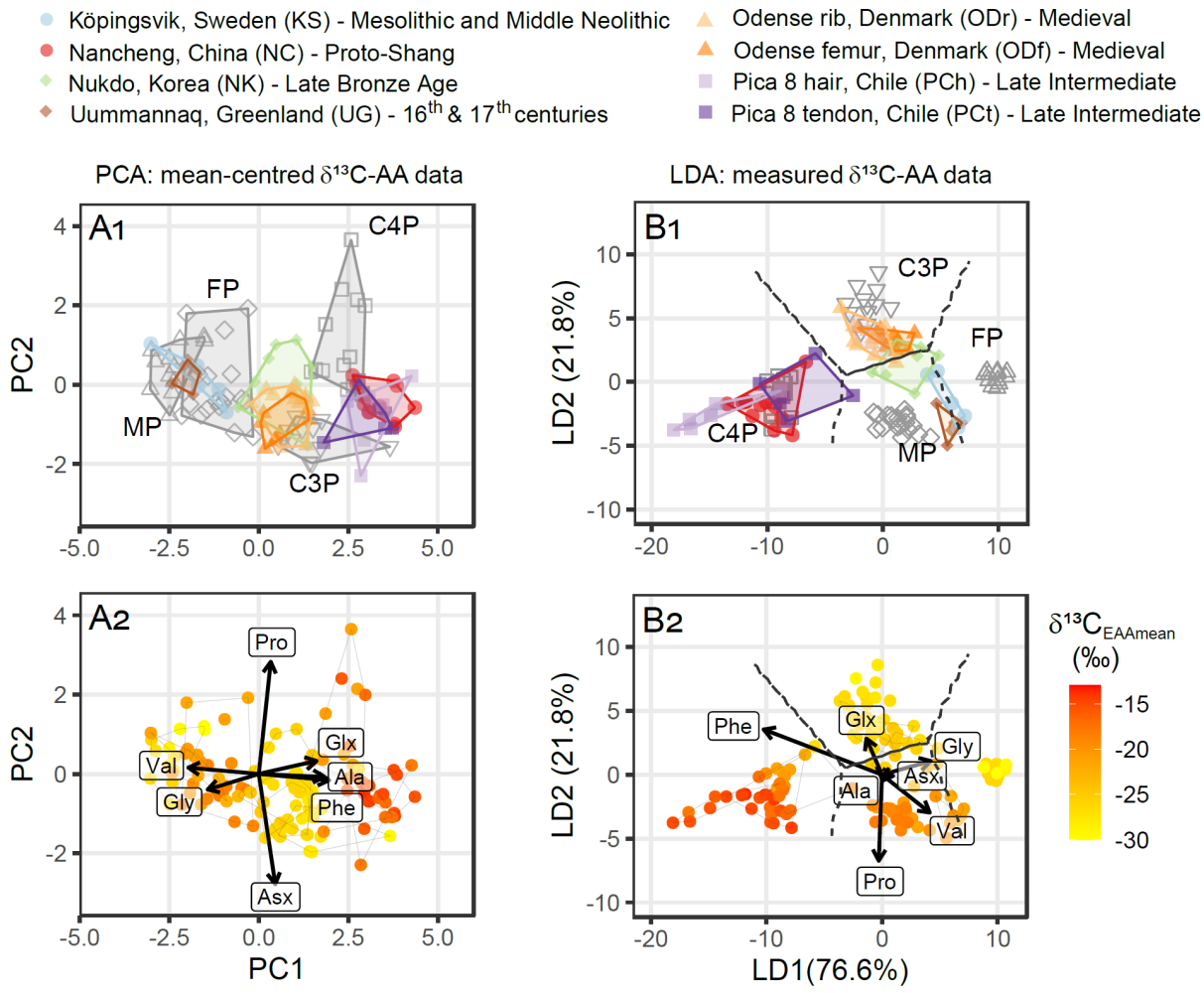

Figure 6. Ordination analyses using $\delta^{13}C$ values of alanine (Ala), aspartate/asparagine (Asx), glycine (Gly), glutamate/glutamine (Glx), phenylalanine (Phe), proline (Pro), and valine (Val) extracted from archaeological human collagen and keratin samples. Subplots A1 and A2 display the first two principal components, based on $\delta^{13}C$-AA values centred to the within-sample mean EAA values (Phe and Val). Subplots B1 and B2 show the first two linear discriminants (LD) based on measured $\delta^{13}C$-AA data. Subplots A1 and B1 categorise individuals according to their respective populations, while the colour-gradient subplots A2 and B2 illustrate variations in individually measured mean $\delta^{13}C$-EAA values. The broken lines in the LDA plots indicate the decision boundaries for freshwater (FP), marine (MP), terrestrial C_3 (C3P), or terrestrial C_4 (C4P) sources based on a subset of individuals with clear archaeological and environmental contexts. These 'known' individuals are denoted with open grey symbols and originate from Belize, Brazil, Bulgaria, Greenland, Guatemala, Japan, Serbia, and Romania (data from Honch et al. 2012, Colonese et al. 2014). Populations with less certain diets are plotted with distinctly coloured symbols and polygons (data from Choy et al. 2010, Raghavan et al. 2010, Mora et al. 2018, Webb et al. 2018, Ma et al. 2021, Brozou et al. 2022). The Odense and Pica 8 populations are based on tissues from the same individuals that reflect short-term (rib and hair) or long-term (femur and tendon) dietary histories. See Appendix S4 for detailed sample information.

6. Considerations for using archival tissues

Changes in basal resource use can occur both within and between populations, and over seasonal to millennial scales. Tracking basal resource use through space and time depends on the rate of AA assimilation in various animal tissues (i.e. tissue turnover rate) and their preservation. Analysing tissues that vary in their AA assimilation rates can indicate consumer basal resource use covering a few days to the entire lifetime of the individual (Boecklen et al. 2011). The ability to reconstruct past basal resource use of individual consumers relies on the preservation and integrity of tissue samples and their δ^{13}C-AA values over time. Natural and artificial preservation have the potential to extend basal resource use reconstructions over several thousands of years under certain conditions. In this section, we discuss tissue characteristics that enable specific basal resource use reconstructions over time and space.

6.1. Temporal resolutions within consumer tissues

The temporal resolution of inferred basal resource use depends on the choice of consumer tissue, as AA assimilation varies across tissue types. Blood and soft tissues, such as liver and muscle, can be turned over completely within days to months depending on tissue metabolism, age or species (Boecklen et al. 2011, Thomas and Crowther 2015, Vander Zanden et al. 2015). Many hard and semi-hard tissues such as bones and ligaments are also remodelled throughout life at different rates varying with age, gender, physiology, and pathological conditions (Hadjidakis and Androulakis 2006). By analysing different skeletal bones, basal resource use reconstruction can span a decade or more (Tieszen 1983, Fahy et al. 2017, Matsubayashi and Tayasu 2019). The complete lifetime can be analysed using additional collagenous tissues that are metabolically inert and therefore not remodelled after formation (e.g. human dentine, Brault et al. 2014), reflecting basal resource use during the period of their formation. Inert keratin excrescences such as hair, nails, and feathers in mammals and birds capture longitudinal basal resource use over seasons as they grow continuously until replaced during moulting. Other keratin tissues such as scales and whale baleen grow incrementally and can be used to reconstruct partial life histories. Entire life histories can be reconstructed from protein incorporated in metabolically inert calcium carbonate structures such as bivalve shells, coral skeletons, and fish otoliths (Payan et al. 1999, Edeyer et al. 2000, Borelli et al. 2001, Falini et al. 2015). As these carbonate structures often form visible incremental bands, temporal subsampling can be performed. Similarly, chitin structures such as cephalopod beaks and cartilage vertebrae of sharks also grow throughout an individual's lifetime displaying incremental bands (Cherel et al. 2009, 2019, Magozzi et al. 2021). However, mechanical structures such as cephalopod

beaks and whale baleen lose material due to wear and therefore their temporal information will be limited to a certain timespan before collection (Aguilar and Borrell 2021).

The temporal resolution gained from incremental biogenic carbonates, chitin structures, and keratin excrescences is dependent on their AA concentration, increment width, and size relative to the sensitivity of the analytical instrument. AA concentrations in shells, fish otoliths and coral skeletons are low, generally ranging between 0.5 and 2% and therefore require larger sample amounts per measurement, although proteinaceous corals have naturally high AA contents (Degens et al. 1969, Williams 2020). Small increment widths in biogenic carbonates may necessitate combining material from multiple increments and thus reduce temporal resolution. Moreover, AA composition can significantly differ between species and tissue types; bones have notably high glycine contents while methionine occurs in low concentrations in many tissues.

6.2 Natural and artificial preservation of tissues

Proteinogenic AAs can withstand high levels of heat, gamma radiation and temperature changes, therefore their preservation largely depends on whether hard tissues are compromised by AA leaching, augmentation or bacterial reworking (Grupe 1995, Collins et al. 2002, Iglesias-Groth et al. 2011). Several degradation indicators such as AA stereoisomer ratios, and stable δ^{13}C-AA and δ^{15}N-AA values and their offsets suggest that high density carbonate matrices such as egg and bivalve shells remain inert for at least 10,000 years under favourable conditions (Tuross et al. 1988, Engel et al. 1994, Silfer et al. 1994, Johnson et al. 1998, O'Donnell et al. 2007). However, unbound protein fractions are prone to leaching and can disappear within the first 6000 years of an organism's death (Bada et al. 1999, Ortiz et al. 2018). External AAs can accumulate on the surfaces of hard tissues and should be removed prior to analysis (e.g. mechanically, by dilute acid washing, or sonication, Engel et al. 1994). However, this can be challenging in porous structures such as coral skeletons and bones where external AAs can be deposited over large internal surfaces (Bada et al. 1999). AAs in lower density matrices such as bones and elastic tissues do not persist on geological timescales except under extremely favourable conditions as humidity and temperature shifts accelerate AA degradation by creating micro-fissures and increasing porosity (Grupe 1995, Maurer et al. 2014). Physical abrasion and leaching can further diminish the protein content of external hard tissues like feathers and fish scales (Salvatteci et al. 2012). Measuring the nitrogen content and atomic ratios of carbon to nitrogen is often standard practice to assess protein preservation in e.g. bones (Brock et al. 2012).

Soft tissues that readily degrade are best preserved either dried or frozen for extensive time periods. However, museums and research institutions often preserve specimens in chemical solvents such as ethanol or formaldehyde solutions. In the short term (<1 year), chemical preservation techniques have no observable effects on δ^{13}C-AA or δ^{15}N-AA values (Strzepek et al. 2014, Durante et al. 2020), but alterations have been observed for samples stored for prolonged periods of up to 27 years (Hannides et al. 2009, Hetherington et al. 2019, Durante et al. 2020, Swalethorp et al. 2020). Beyond this, it is unclear how solvents affect δ^{13}C-AA values in proteinaceous tissues, but storage over centennial timescales or heating causes tissue disintegration and loss of AAs to the surrounding solvent (Von Endt 2000, Marte et al. 2003). It is likely that the preservation chemicals affect tissue integrity by impacting the peptide and protein bonds. This could lead to unstructured AA leaching into the surrounding fluids in the long term, affecting δ^{13}C-AA values of the tissue due to mass-based diffusion differences. To fully embrace δ^{13}C-AA analysis of chemically preserved tissues, further experimental investigations into the potential effects of chemical preservatives on δ^{13}C-AA values are warranted.

7. Minimising analytical uncertainties in δ^{13}C-AA values

Carbon isotope analysis of AAs poses greater methodological challenges and potential for analytical errors than bulk stable isotope analysis. Bulk isotope analysis consists of weighing dried tissue that is then combusted in the elemental analyser, although some samples may require lipid or mineral removal prior to analysis. The procedures for AA analysis are more intricate as AAs must be extracted and isolated from the diversity of compounds within the sample (see Figure 7). Isotopic measurement can be done using a gas chromatograph interfaced to a combustion reactor and isotope ratio mass spectrometer (GC-IRMS) or with a liquid chromatograph (LC-IRMS). For GC-IRMS, polar charged AAs need to be chemically modified to enable their evaporation (Silverman et al. 2022). With LC-IRMS, AAs can be directly analysed after isolation but analytical sensitivity is comparatively low and therefore less commonly employed (Smith et al. 2009, Dunn et al. 2011). Monitoring the consistency and stability of compound-specific isotope measurements further requires the extensive use of reference materials. Since there are diverse approaches to analytical protocols, instrumentation, and referencing between laboratories that can affect the accuracy of δ^{13}C-AA measurements, here we discuss achieving analytical consistency and inter-laboratory comparability when measuring δ^{13}C-AA values.

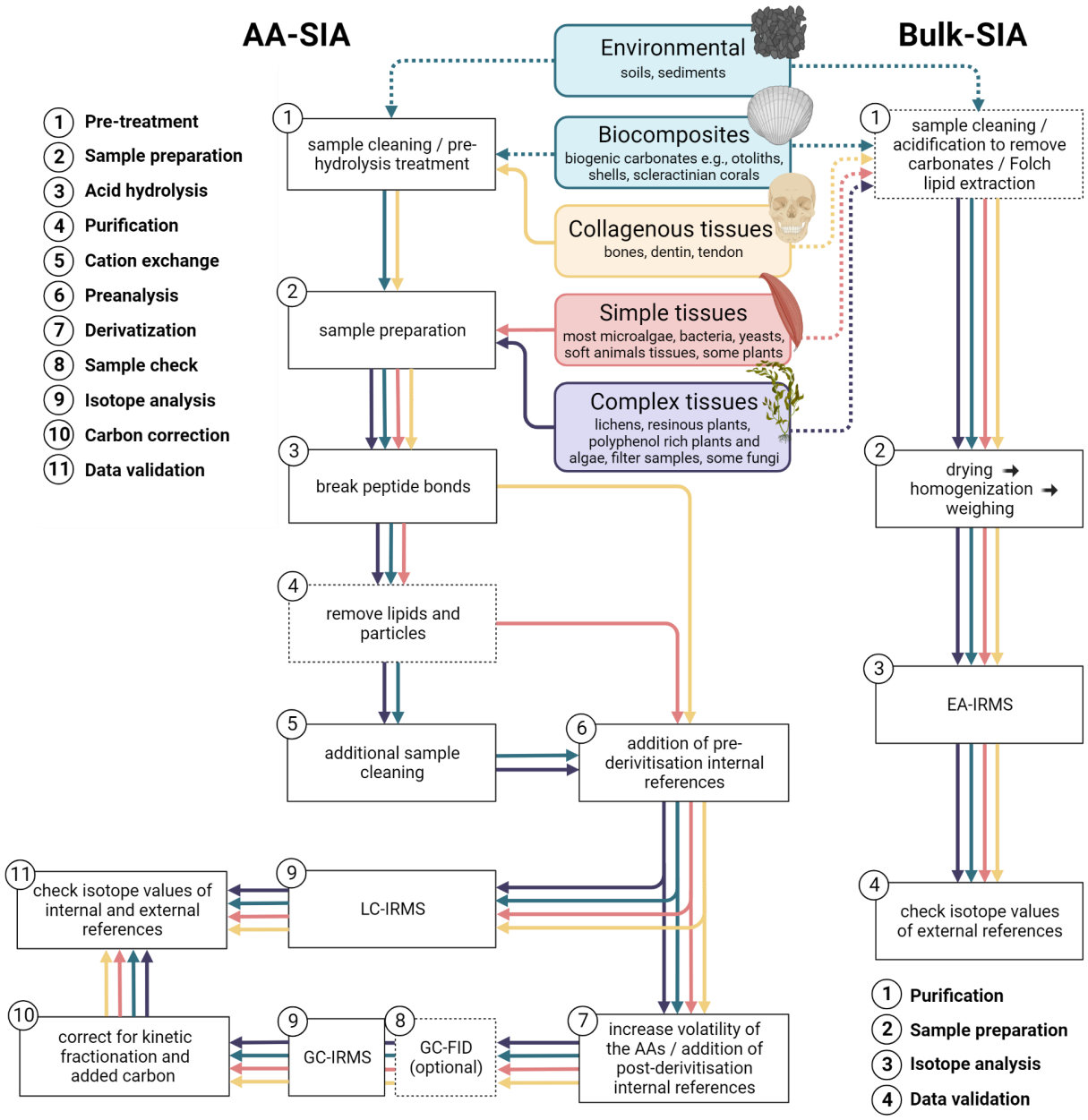

Figure 7. Analytical protocol workflows for AA stable isotope analysis of different sample types (coloured boxes and corresponding arrows) contrasted against bulk stable isotope analysis. Broken line arrows and boxes indicate that the treatment step is not mandatory for all samples.

7.1. Analytical workflow

To assess the stable isotope composition of individual AAs, they must first be extracted and isolated from other compounds in the sample. Typically, this involves drying and homogenising the samples, followed by acid hydrolysis where strong hydrochloric acid and high temperatures break down proteins and

peptides into their individual AAs (Figure 7: step 2 and 3, Enggrob et al. 2019). However, acid hydrolysis also disrupts other chemical bonds, including within the EAA tryptophan, yielding a complex mixture of AAs among other organic molecules and salts. The removal of the non-AA fraction, or purification, is essential as it interferes with later steps in the analytical workflow and isotopic measurements. Purification steps vary between analytical facilities and depend on the type of tissue analysed and chemical protocol employed. To analyse AAs in bone collagen, bone carbonate AAs need to be removed as they are more susceptible to diagenetic processes and have different turnover times than collagen (Stafford et al 1988, Lambert and Grupe 1993). Thus, bone carbonates are removed by soaking whole bones in a light acid (Figure 7: step 1, Sealy et al. 2014), while other biogenic carbonates are generally homogenised, acid hydrolysed, and purified with cation exchange resins (Figure 7: step 5). For samples rich in secondary metabolites, such as those from soils or plant-algal sources, cation exchange resins may also be necessary. Large particulates remaining in the hydrolysed samples can be removed with glass wool filtration, whereas excessive lipophilic compounds can be removed through chemical extractions (Figure 7: step 4). After purification, samples are dried and internal reference compounds that are molecularly similar to AAs can be added to account for any potential AA losses or isotope effects (Figure 7: step 6). Before undergoing GC-IRMS analysis, the AAs are chemically modified - known as derivatization - to make them more volatile and enable chromatographic separation. This process is done by reacting AAs with reagents that specifically target AA functional groups (Figure 7: step 7). Following derivatization, it is common to introduce additional internal reference compounds like caffeine with known isotope values. Once the derivatized AAs are analysed via GC-IRMS, the resulting chromatograms must undergo quality control and assurance as the automatic AA peak integration may be incorrect and peaks may overlap (i.e. co-elute; Figure 7: step 9). Co-elution between AAs and non-AA compounds leads to errors in isotope measurements when an AA peak incorporates another compound's lighter ^{12}C peak start or heavier ^{13}C peak tail (Meier-Augenstein 2002, Sessions 2006). Because the derivatization process incorporates external carbon into the AAs, acquired ^{13}C-AA data need to be corrected using mass-balance equations and predefined isotope correction factors (Figure 7: step 10, Docherty et al. 2001). The long-term stability of AA carbon isotope measurements and instrument performance should be monitored by running external reference compounds with known isotope values (Figure 7: step 11).

7.2. Pitfalls in the analytical workflow

Sample pre-treatments and purification protocols vary widely in their complexity and scope (Figure 7: step 1, 4, 5), but whether they bias δ^{13}C-AA values is not always known. Acidic treatments and chemical extractions followed by extensive water rinsing are often employed to remove minerals, urea, and lipids in animal consumer tissues, despite being discouraged as they lead to large and inconsistent isotopic measurements (Brodie et al. 2011, Schlacher and Connolly 2014, Pellegrini and Snoeck 2016, Huang et al. 2023). Such pretreatments may result in the loss of AAs and alter the δ^{13}C-EAA values in consumer tissues compared to dietary tissues that are often left untreated (see Appendix S5). We compiled 17 controlled feeding studies to highlight the potential isotopic effects of aqueous pretreatments. Data reveal inconsistent changes in δ^{13}C-EAA values between diets and consumer tissues, ranging from -13 to 12‰, in studies utilising extensive water rinses and acidic pretreatments (Figure 8, Appendix S5). In contrast, non-aqueous pretreatment studies report values consistently much closer to 0‰ (Figure 8, Appendix S5). Applying aqueous pretreatments to soft tissue samples may wash away small peptides and free AAs by dissolving hydrophilic proteins and AAs. While more studies are warranted to investigate this potential issue, we recommend post-hydrolysis purification methods, such as cation exchange or solid-phase extraction, as alternatives that are demonstrably less bias prone (McMahon et al. 2010, Takano et al. 2010, Ohkouchi et al. 2017). This is similarly the case for samples containing calcium carbonate ($CaCO_3$), which during acid hydrolysis is converted to calcium chloride ($CaCl_2$), a compound which readily absorbs water. Water-sensitive derivatization reagents, such as acetyl chloride and acetic anhydride, react with the water and form compounds that can co-elute with the AAs during chromatography. These issues can be mitigated by using post-hydrolysis purification with cation exchange resins only, or using water-insensitive reagents (e.g. methoxycarbonyl esterification [MOC], Walsh et al. 2014, Vane et al. 2018).

Adding exogenous carbon during AA derivatisation requires careful calculation to offset any changes in δ^{13}C values. While mass-balance equations and predefined isotope correction factors can help, they are not fail-proof (Figure 7: step 10, Docherty et al. 2001, Takizawa et al. 2020). Using derivatisation reagents whose δ^{13}C values closely match sample values and ensuring that reactions go to completion helps minimise errors. Methods like MOC and N-acetyl methylation (NACME) are advantageous in this regard, as they introduce limited additional carbon and provide stable derivatized AAs that can be stored for extended periods (Corr et al. 2007, Walsh et al. 2014). Care needs to be taken when drying samples following derivatisation as drying times can vary substantially between sample types. Yet excessive

over-drying may lead to the partial evaporation of low molecular weight AAs due to their increased volatility.

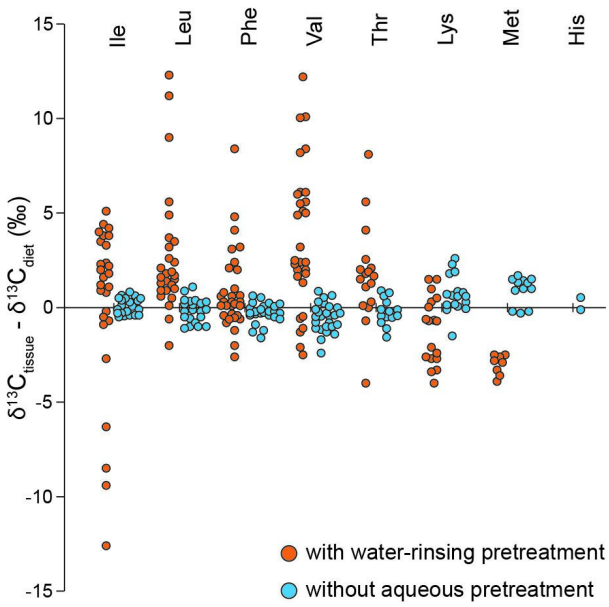

Figure 8. The differences in measured $\delta^{13}C$ values for individual EAAs, isoleucine (Ile), leucine (Leu), phenylalanine (Phe), valine (Val), threonine (Thr), lysine (Lys), methionine (Met), histidine (His), observed between diet and consumer tissue in 17 controlled feeding studies divided on the use of water-rinsing in the purification of consumer tissue samples. No distinction is made between the various consumer tissue types (muscle, intestine, heart, liver, bone collagen, blood plasma, eggshell) or diet qualities (C_3/C_4 origins, macronutrient composition, and prey organisms). See Appendix S5 for specific details on individual studies.

7.3. The need for standardisation

Maintaining the integrity of $\delta^{13}C$-AA measurements requires careful oversight, especially for inter-laboratory consistency and temporal stability. Robust chromatographic practices, such as complete peak separation and Gaussian peak shapes, are fundamental for accurate isotopic measurements, as co-elution biases individual $\delta^{13}C$-AA measurements (Meier-Augenstein 2002, Sessions 2006). Monitoring instrument stability and accuracy, i.e. measurement standardisation, is achieved through the use of internal and external reference compounds (Meier-Augenstein and Schimmelmann 2019). Internal references are added directly to the sample (Figure 6: steps 6 and 7) and provide immediate calibration, track potential isotope effects, and monitor AA losses. Estimating the required concentrations of internal references to be added can be challenging due to the often uncertain and low AA concentrations in samples, although sample AA concentrations could be assessed, for example, by GC with Flame

Ionization Detection (GC-FID, Figure 7: step 8). External references run separately serve as benchmarks (Figure 6: step 11), and should cover the range of $\delta^{13}C$ values encountered in the samples. External references can be subdivided into two categories: derivatized AAs with known isotopic values (Roberts et al. 2018, Meier-Augenstein and Schimmelmann 2019) and non-derivatized compounds, e.g. caffeine, fatty acid methyl esters or *n*-alkanes. The former account for isotope effects introduced during derivatization, while the latter calibrate the reference CO_2 monitoring gas and provide a long-term stability check for $\delta^{13}C$ values (Schimmelmann et al. 2016).

To address and reduce biases arising from diverse analytical protocols, equipment, and sample matrices across different research facilities, researchers must implement a practice of thoroughly detailing their methodological protocols in publications, as proposed by Dunn and Skrzypek (2023). Simultaneously, there is a pressing need for accessible biological reference materials, part of a wider call in isotope analysis generally (e.g. Stichler 1995, Gröning 2004, de Laeter 2005). Selecting suitable references for $\delta^{13}C$-AA analysis is challenging however: the materials must be homogeneous, easy to transport, and ideally neither hazardous nor biologically active. The availability of such international reference materials would bolster the reliability and comparability of $\delta^{13}C$-AA data across studies. Standardising methodologies (Figure 7) would further enhance this, reducing the additional biases introduced by the array of protocols and chemicals currently in use, improving the inter-comparability of values measured in different facilities, and enabling a $\delta^{13}C$-EAA fingerprint library (section 4.1).

8. From qualifying to quantifying basal resource use

Consumer tissue $\delta^{13}C$-EAA patterns are a composition of the $\delta^{13}C$-EAA patterns of the assimilated basal organisms. The analysis of compositional data has a long history, spanning geology with the analysis of mineral composites (Weltje et al. 1997) to remote sensing where incoming spectra are mixtures of pure spectral components (Clevers and Zerita-Miller 2008). The statistical framework used to estimate proportional contributions in compositional data is a linear (un)mixing model (Weltje et al. 1997, Phillips 2012, Parnell et al. 2013). Over the past 20 years, significant development of mixing models has addressed many issues associated with biological systems. These include complex data structures that impart variation in basal resource use in individual consumers (Semmens et al. 2009, Stock et al. 2018); the multitude of potential basal resource combinations that could result in the same $\delta^{13}C$-EAA patterns (known as under-determined mixing systems, Parnell et al. 2010); and natural variations and

measurement errors in resources and consumers (Moore and Semmens 2008, Stock et al. 2018). Here we outline the application of mixing models with δ^{13}C-EAA data, highlighting key considerations, assumptions and limitations. While several implementations of mixing models are available (Wang et al. 2019b, Cheung and Szpak 2021, Heikkinen et al. 2022), we primarily focus on the MixSIAR package in *R* (Stock et al. 2018) due to its flexibility and relatively common use across ecology.

8.1. Consolidating basal resource information

The area bounded by basal organism δ^{13}C-EAA fingerprints constitutes the mixing space: the area containing all possible consumer tissue δ^{13}C-EAA patterns (Phillips et al. 2014, Smith et al. 2013). The dimensionality of this mixing space is equal to the number of tracers: the number of EAAs measured. All potential basal resources that may contribute to consumer δ^{13}C-EAA patterns should be characterised (see section 3.2), as the proportional contributions of basal resources are not independent of each other: they must, by definition, sum to one. Missing basal resources is a general problem when resolving linear mixing systems (Weltje et al. 1997), resulting in inaccurate proportions regardless of the statistical approach. Consumer δ^{13}C-EAA data falling outside of the mixing space may indicate missing resources. However, even if consumers fall within the basal resource mixing space, it is still possible that some utilised basal resources have not been characterised.

While uncharacterised basal resources are problematic, it is important to limit basal resources to only those that likely contribute to consumer tissues. While it may seem reasonable to include as many basal resources as possible, an underlying assumption of mixing models is that all included basal resources contribute to the consumer δ^{13}C-EAA values, even if that contribution is small. Excluding unused basal resources limits model complexity, aiding model performance, and improves model accuracy by removing isotopically feasible but biologically unrealistic combinations. Further, statistical artefacts arise when resolving mixing models with high numbers of basal resources as solutions will tend towards 1/n for large n. Therefore, it is recommended to limit mixing models to seven or fewer basal resources (Stock et al. 2018). Following characterisation, any modifications between basal resources and the consumer tissues need to be defined, known as trophic discrimination factors (TDFs). Although TDFs need to be considered for many types of tracers (Schulting et al. 2022), they are negligible for δ^{13}C-EAA data.

The natural variation in basal resource δ^{13}C-EAA values needs to be considered in mixing models, but can be inadequately described when logistical and analytical constraints result in low sample sizes. Although

uncertainty due to low sample sizes can be incorporated into Bayesian mixing models, it reduces model precision. Basal resource δ^{13}C-EAA variability could be approximated using well-constrained literature sources, however differences in methodologies and analytical processes, and the absence of international reference materials will add additional uncertainties (section 7). Uncertainty in instrumental measurements is rarely considered when quantifying basal resource use with δ^{13}C-EAA data (Hopkins and Ferguson 2012; but see Vane et al. 2023). Mixing model frameworks initially developed for bulk stable isotope analysis are based on data with limited instrumental error (typically 0.1-0.2‰ for bulk δ^{13}C and δ^{15}N). Analytical uncertainty in δ^{13}C-EAA values can be larger (~1‰) and AA specific, and should be incorporated into mixing models to ensure uncertainty estimates are not artificially deflated.

8.2. Modelling consumer behaviour

Specific hypotheses regarding consumer basal resource use inform how mixing models are structured. Factors that contribute to differences in basal resource use between consumers should be incorporated into the model. Examples include species, sex, size or ontogenetic stage, social status and other traits that result in different nutritional requirements or limit access to specific dietary items. For example, body size can limit access to particular habitats or lower social status could restrict access to nutrient-dense foods such as meat. Hierarchical spatial structuring of consumers such as distinct subpopulations within larger areas or spatially discrete sampling sites should be considered. Spatial structure can affect basal resource availability and use, even if preferences are the same among individual consumers (Semmens et al. 2009), and similarly applies to consumers sampled in different time periods (e.g. seasons, years).

Bayesian mixing models can incorporate prior information to inform model solutions, for example basal resource use estimates extracted from mass-balanced food web models (Stock et al. 2018). However, prior information can be biased (e.g. stomach and scat data towards poorly-digestible prey), and overly restrict mixing model solutions (Swan et al. 2020). Theoretically, known nutritional limitations such as macronutrient requirements can be included as prior information where consumers have considerable diversity in diet quality. However, prior information typically pertains to consumer diet (i.e. the proportions of prey assimilated) rather than basal resource use, and therefore should be considered carefully with δ^{13}C-EAA data.

Error structures are often overlooked in mixing models. For groups of consumers, residual errors in MixSIAR are modelled as a multiplicative term (called a residual stretch error), rather than Gaussian noise, that stretches or compresses the variance attributed to model processes (stochastic sampling of basal resource variation and additional uncertainties, Stock et al. 2016, 2018). This is conducted separately for each tracer. The ecological justification is that consumers feed many times during basal resource assimilation, dampening the isotopic variation observed in basal resources. This contrasts with mixing models that sample basal resource δ^{13}C-EAA values from their distributions only once when estimating model solutions. Residual stretch errors are therefore expected to take values between 0 and 1 to compress variation due to feeding behaviours. Values approaching zero can be interpreted as an increase in the number of feeding events reflected in the consumer tissue, synonymous with a slower tissue turnover rate. Values in excess of one indicate that factors beyond those included in the mixing model are driving individual variation. For passive trophic behaviours such as sessile filter-feeding or grazing, the stretch error approach works well (Stock et al. 2016). However, active and selective feeding modes in motile consumers may violate the assumption of stochastic source sampling across individuals, inflating residual stretch error estimates. In such instances it may be more appropriate to incorporate individuals as an additional random effect in the model structure. The drawback of this approach is that all residual intra-group variation in δ^{13}C-EAA values is solely attributed to differences in individual basal resource use. In reality, most systems comprise some degree of individual variation in basal resource use, and other undefined sources of isotopic variation. While the suitability of different error structures can be explored in terms of model goodness of fit (Cheung and Szpak 2021), emphasis should be given to the biological interpretations and their trade-offs within the studied system.

8.3. Interpreting mixing model output

Underpinning a mixing model's ability to accurately estimate basal resource use by the consumer is the separation between basal resources. It is necessary to first check whether basic model assumptions are met, the model has converged, and the optimal model structure has been determined (see Phillips et al. 2014). If the δ^{13}C-EAA patterns of two basal resources cannot be distinguished, i.e. are not δ^{13}C-EAA fingerprints, then this results in a strong negative correlation between the proportional contributions of those two basal resources and potential bimodality in their posterior distributions (Phillips et al. 2014). In such cases the proportional contributions of the two basal resources should be combined into a single group post analysis. Although basal resource distinction is lost, often it will drastically reduce the overall

uncertainty in proportional contributions (Phillips et al. 2014). Similarity between basal resource δ^{13}C-EAA patterns is often tested statistically by comparing the mean δ^{13}C-EAA offsets of basal resources for each AA separately. However, equality of means testing depends on large sample sizes to be robust (Stock et al. 2018), which is typically not the case for δ^{13}C-EAA data, and does not consider differences in variances and covariances between basal resources. If required, statistical scrutiny should be conducted using a multivariate approach (e.g. Bhattacharyya coefficients, Figure 4) that quantifies the similarity of multidimensional distributions. If two or more basal resources are isotopically similar, it is still recommended that their proportional contributions be combined post analysis rather than merging them prior to implementing the mixing model (Stock et al. 2018). In some cases, all basal resources may exhibit similar means across one or more EAAs. It may seem logical to remove such tracers to reduce model complexity and aid model convergence. However, users could unintentionally be removing AA tracers that help mixing models to resolve by reducing information on differences in basal resource variances or covariances. Increasing the number of EAAs in mixing models maintains or reduces overall model uncertainty, therefore all available EAAs should be included in mixing models. This has been demonstrated for δ^{13}C-EAA data with mice fed varying diets, with mixing models including all measured EAAs providing the most accurate solutions across diets with reduced uncertainties compared to those using a restricted set of AAs and other statistical approaches (Manlick et al. 2022).

The trade-off in attributing intra-group variability in consumer tissue δ^{13}C-EAA values to consumer-resource sampling processes (with residual stretch errors) or differences in individual basal resource use (section 8.2) should be considered carefully. Stretch errors can identify whether one or more basal resources are insufficiently characterised or indicate other issues with model components, e.g. analytical uncertainty. Stretch errors slightly greater than one are not necessarily suggestive of poor model quality: many complex biological and ecological processes impart variability that cannot be measured or captured in statistical models. However, stretch error values that are much greater than one can indicate that one or more substantive processes are lacking from the mixing model. If stretch errors are inflated for many to all of the EAA tracers, then this likely indicates missing but significant driver(s) of basal resource use from the model structure. If only one or a few EAA tracers have inflated stretch errors, then more EAA tracer-specific sources of variation need to be identified. This could include a missing basal resources that significantly differ in δ^{13}C values for the identified EAAs (Vane et al. 2023) or poorly constrained EAA specific variations. While such situations may be problematic for testing specific hypotheses, they can be useful in highlighting inadequacies in current knowledge.

A mixing model's ability to partition basal resource use with precision ultimately depends on the mixing space, the positions of basal resources and consumers within it, and their uncertainties. Robustly quantifying basal resource use can therefore be highly ecosystem-specific. If consumers depend on only a few, isotopically similar basal resources, then their δ^{13}C-EAA pattern mixing area will be small, increasing uncertainty in model estimates. This can be exacerbated if other sources of uncertainty, such as measurement errors for individual EAAs, are large. Small signal to noise ratios in δ^{13}C-EAA data are reflected in large uncertainties in mixing model solutions, capturing the true uncertainty associated with disentangling basal resource use. In such instances, using measured δ^{13}C-EAA values may prove fruitful where strong environmental gradients separate basal resources, but comes with greater logistical restraints (section 5).

8.4. Considerations when quantifying basal resource use

Quantitative approaches using δ^{13}C-EAA data provide complementary benefits but additional complexities compared to bulk stable isotope data. δ^{13}C-EAA data are well suited to trace the flow of basal resource biomass through food webs due to their direct routing into consumer tissues. However, this contrasts with the theoretical underpinning upon which ecological mixing models were developed: quantifying proportional contributions of prey-specific biomass assimilated into consumer tissues (i.e. consumer diet). The nuanced differences between basal resource use and diet need to be considered during study design, sample collection, and data analysis to avoid erroneous inferences.

The application of extensive training datasets is becoming commonplace in δ^{13}C-EAA studies (e.g. Arsenault et al. 2022b, section 4.1). However, such training datasets result in inflated variation and potential bias (mean offsets) in δ^{13}C-EAA patterns, preventing the characterisation of δ^{13}C-EAA fingerprints compared to in situ sampling (section 4.1 and 7.2). This is highlighted in Figure 9 where we show how the variability in a training dataset compares to specific in-study sampling of basal resources in LDA space. Mean δ^{13}C-EAA pattern bias can be observed for several basal resource groups, notably fungi, and inflated variation means study-specific fingerprints are lost. Mixing models are sensitive to input data (Bond and Diamond 2011), so applying mixing models with broad training datasets increases uncertainty in mixing model solutions and could lead to false inferences (Manlick and Newsome 2022). In some instances, logistical constraints can limit complete basal resource characterisation, necessitating the use of carefully selected external data. However, while international reference materials are still

lacking, external data should not be used as a substitute for adequate system sampling when the aim is to accurately quantify basal resource use.

There are many underlying conditions and assumptions for robust proportional estimations with mixing models. Consequently, other semi-quantitative techniques have been implemented to resolve mixing systems, notably bootstrapped LDA-based classifications (e.g. Fox et al. 2019, Skinner et al. 2021, Arsenault et al. 2022b). Arguments for this approach include a “less rigid framework” regarding uncharacterised basal resources and multivariate mixing space geometry (Fox et al. 2019, Manlick and Newsome 2022). Such arguments misconstrue that the “rigid” assumptions are inherent to the Bayesian mixing model methodology rather than mixing systems themselves. For example, individual consumer data falling outside of the basal resource mixing space implies an inadequately described mixing system. This general problem can be masked by LDA dimensionality reduction, but is more noticeable when implementing Bayesian mixing models. Recent simulations on lake ecosystem data highlight that significant bias can occur between known basal resource contributions and those estimated using this LDA approach (Saboret et al. 2023). Moreover, we argue that the perceived limitations of mixing models should be considered a strength in that they require adequate prior understanding of the ecosystem and consumer (Makarewicz and Sealy 2015). It is frequently highlighted that mixing models are only as good as the input data (Phillips et al. 2014), yet they are also only as good as our understanding of ecosystem processes that can be described using mathematical abstraction.

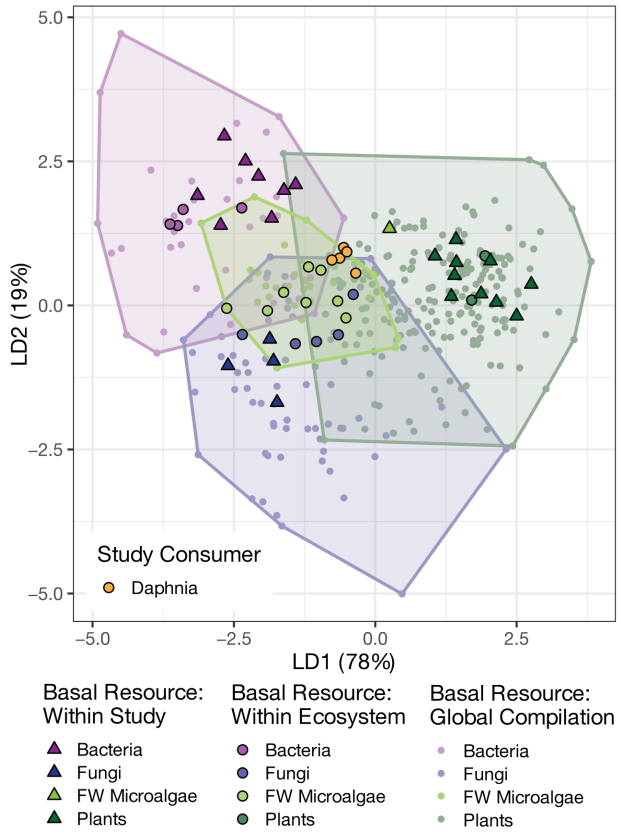

Figure 9. LDA plot highlighting the increased variation and mean bias introduced to basal resource δ^{13}C-EAA patterns when using training datasets (individual data points plus their convex hulls, Figure 4) compared to within study sampling designs. Data taken from a water flea *Daphnia* sp. study in Alaskan lakes (Larsen et al. 2013). CAM plants and extremophile bacteria were excluded as they do not contribute to the study ecosystem (section 3.1). Within-study collected basal organisms are plotted as triangles, whereas cultured/sampled basal organisms from other ecosystems are plotted as circles (reported in Larsen et al. 2013 as similar ecosystems). Within-study freshwater (FW) microalgae consists of a single seston filtrate composite that falls outside of the compilation data, and is likely a mixture of microalgae and other allochthonous POM.

9. Perspectives on δ^{13}C-AA applications in food web ecology

Carbon stable isotope analysis of AAs represents a considerable development in the analytical tools for tracing basal resources in food webs. Richly layered δ^{13}C-AA datasets offer detailed insights into the intertwined trophic, metabolic and environmental processes that obscure interpretations in traditional bulk stable isotope approaches (Yun et al. 2022). With spatiotemporally consistent δ^{13}C-EAA fingerprints, coupled with the stability of AAs in well-preserved tissues, reconstructions of consumer basal resource use can extend into the paleontological record. Baseline δ^{13}C-EAA values incorporate environmental effects, providing inferences about the basal organism habitat while δ^{13}C-NEAA values extend insights to include dietary macronutrient content, diet quality, and catabolic processes in consumers. Given the

diverse metabolic roles of AAs, δ^{13}C-AA data help infer the metabolic processes that underpin cellular and tissue functioning, unlocking valuable inferences into the dynamic nutrient flows and physiological responses that shape ecosystems. Diverse aspects of basal resource use in food webs can therefore be investigated with δ^{13}C-AA data when the underlying mechanisms of δ^{13}C-AA values are sufficiently understood.

A priori ecological knowledge informs study sampling specificity, and the subsequent robustness of inferences made from δ^{13}C-AA data. The high taxonomic resolution reflected in the δ^{13}C-EAA patterns of basal resources is becoming increasingly apparent, notably within plants and algae (section 3.1, Scott et al. 2006, Larsen et al. 2020, Vane et al. 2023, Stahl et al. 2023). Although exhibiting equally diverse δ^{13}C-EAA patterns, a lack of data impedes comprehensive analyses of δ^{13}C-EAA pattern specificity within bacteria and fungi. Further development of the mechanistic underpinning of δ^{13}C-EAA patterns in basal organisms, as initiated here in sections 2 and 3, would facilitate targeted analyses of discriminatory resolution. Importantly, ecological distinctions are not necessarily reflected in δ^{13}C-EAA patterns, for example discerning between fresh and detrital material of basal organisms can be challenging (Vane et al. 2023) due to δ^{13}C-EAA patterns remaining consistent during tissue necrosis, fragmentation, and detrital transport (Larsen et al. 2013, Elliott Smith et al. 2022).

Extending beyond discriminating basal organisms and reconstructing basal resource use in consumers, harnessing the trove of metabolic information embedded in δ^{13}C-AA data relies on a solid mechanistic understanding of the processes that contribute to individual AA carbon isotope values. While progress has commenced in recent years (e.g. Larsen et al. 2015, Manlick et al. 2022, Elliot Smith et al. 2022, Stahl et al. 2023), this has been outpaced by the broad and expanding applications of carbon stable isotope analyses of AAs in the literature. Viewing δ^{13}C-AA data through a physiological lens can help generate new hypotheses, such as the synthesis of ^{13}C deplete lignin resulting in relatively enriched δ^{13}C values of phenylalanine in vascular plants (section 3.1). Furthermore, δ^{13}C-AA data could shed light on the degree of direct AA incorporation in facultative prototrophs. Culturing basal organisms on AA-free media establishes the δ^{13}C-AA pattern of purely de novo synthesised AAs, which can be compared to those sampled in situ, revealing the degree to which external AAs are directly assimilated into the proteins of facultative AA prototrophs in natural settings. Such insights would detail the biochemical functioning of saprotrophic communities, disentangle the metabolic roles of heterotrophy in mixotrophs, and could be facilitated by the development of position-specific stable isotope analyses (Fry et al. 2023).

Despite the richness δ^{13}C-AA data provides, its increased analytical effort has likely contributed to the trend of incorporating external training data into study designs, varying from graphical comparisons (e.g. Stahl et al. 2023) to extensive training data within mixing models (e.g. Arsenault et al. 2022b). Herein lies, however, the often underappreciated issue of interlab comparisons, a problem that is not unique to the carbon stable isotope analyses of AAs (e.g. Stichler 1995, Gröning 2004, de Laeter 2005). Direct δ^{13}C-AA data comparisons would be facilitated by greater standardisation of analytical methodologies across research facilities (Figure 7). Studies comparing inter-lab methodologies could pinpoint the specific processes within protocols that cause measurement biases, improving our understanding of stepwise fractionations associated with specific workflows and redressing issues with incorporating externally derived data into study designs. Ultimately, δ^{13}C-AA values could be collated into a single, taxa-specific reference library for future studies, constituting a separate functional role to the wider calls for a centralised repository for isotope data (Pauli et al. 2017).

The application of δ^{13}C-EAA fingerprints holds immense potential for addressing pressing ecological questions on changing productivity in food webs. The δ^{13}C-EAA fingerprinting approach affords the opportunity to explore carbon fluxes across spatiotemporal scales without having to characterise changes in baseline δ^{13}C-EAA values, offering basal resource characterisation and tracing that is unparalleled in its specificity and inclusivity. Questions such as whether consumers have adapted to the anthropogenic changes in their environment by changing specific basal resource use have scarcely been explored. Similarly, detailed changes in basal resource use across consumer ontogeny, over seasons and years, or between populations remain yet to be thoroughly explored. Understanding basal resource use by metazoans and their physiological responses in conjunction with changes in basal resource abundance, composition, nutritional quality, and the environment provides valuable insights into the resilience of differing food webs across the world.

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Appendix S1

The power and pitfalls of amino acid carbon stable isotopes for tracing origin and use of basal resources in food webs

Vane K., Cobain M.R.D., Larsen T.

Ecological Monographs

Three detailed figures of metabolic networks in plant, heterotrophic bacteria, and animal cells

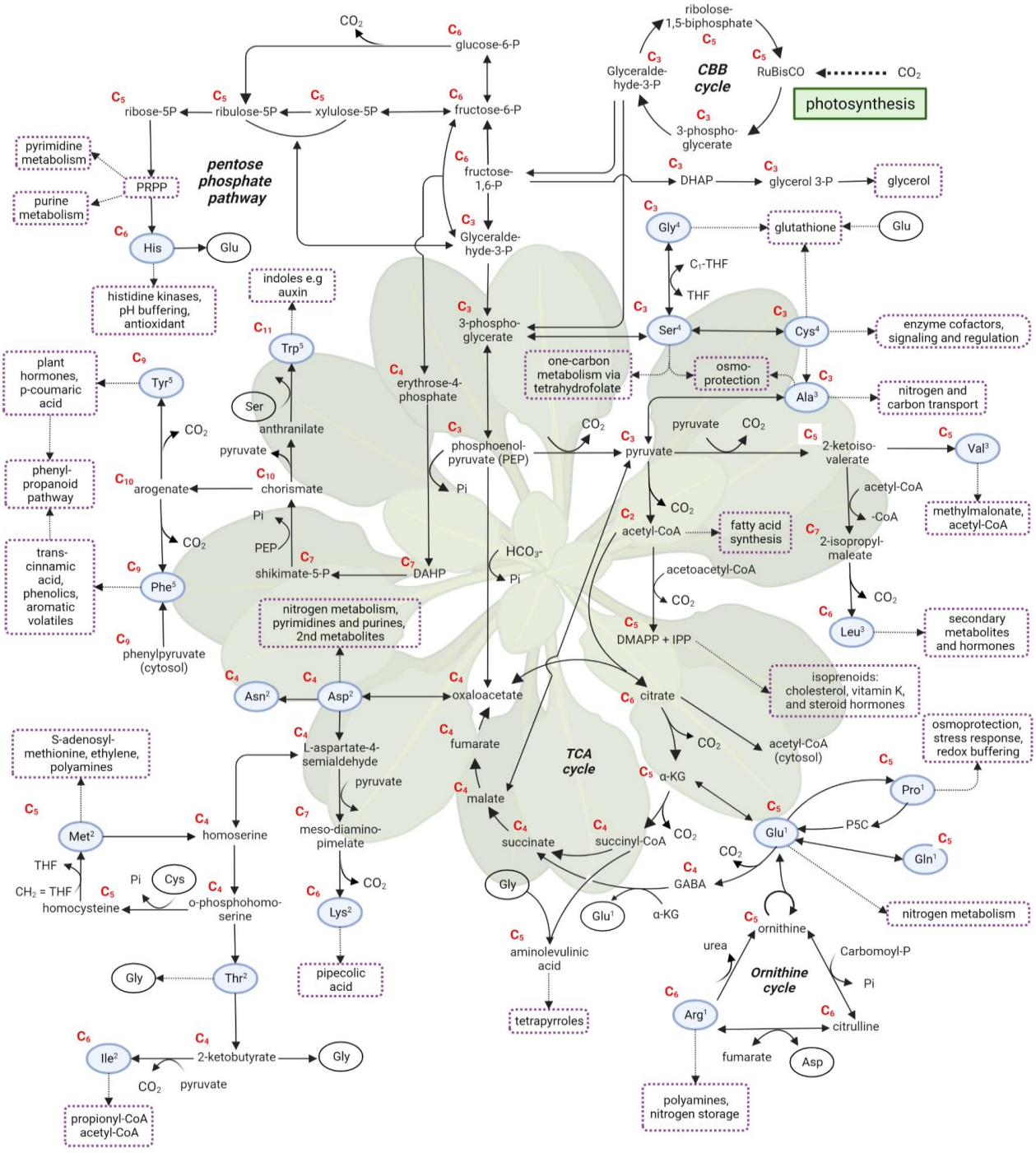

Figure S1A. Amino acid biosynthesis pathways in plants. Simplified schematic overview of the anabolic and catabolic amino acid (AA) pathways in plants, using *Arabidopsis thaliana* as a model species. Based on chemical similarities and precursors, the AAs can be categorized into five groups: 1) the glutamate family, originating from alpha-ketoglutarate (α-KG); 2) the aspartate family, originating from oxaloacetate; 3) the pyruvate group; 4) the 3-phosphoglycerate group; and 5) the aromatic group, derived from phosphoenolpyruvate and erythrose-4-phosphate. Superscript numbers next to each AA indicate its categorization, and filled ellipses represent products of the primary biosynthesis pathway. In addition to serving as structural components in proteins, AAs fulfill a wide range of biological roles, functioning as metabolites, energy-yielding substrates, and signaling molecules, as indicated by the descriptions within the rounded rectangles. Abbreviations: Ala, alanine; α-KG, alpha-ketoglutarate; Asn, asparagine; Asp, Asparagine; CBB, Calvin-Benson-Bassham; Cys, cysteine; DAHP, 3-deoxy-D-arabinoheptulosonate 7-phosphate; DMPP, dimethylallyl pyrophosphate; GABA, γ-Aminobutyric acid; Gly, glycine; Gln, glutamine; Glu, glutamic acid; His, histidine; Ile, isoleucine; IPP, Isopentenyl pyrophosphate, Leu, leucine, Lys, lysine; Met, methionine; P5C, 1-pyrroline-5-Carboxylate; Phe, phenylalanine; Pro, proline; PRPP, Phosphoribosylpyrophosphate; RuBisCo, ribulose-1,5-bisphosphate carboxylase-oxygenase; Ser, serine; TCA, tricarboxylic acid; Trp, tryptophan; Tyr, tyrosine; Val, valine. The pathways are based on the KEGG PATHWAY database (https://www.kegg.jp/kegg/pathway.html). The illustration was created with BioRender.com.

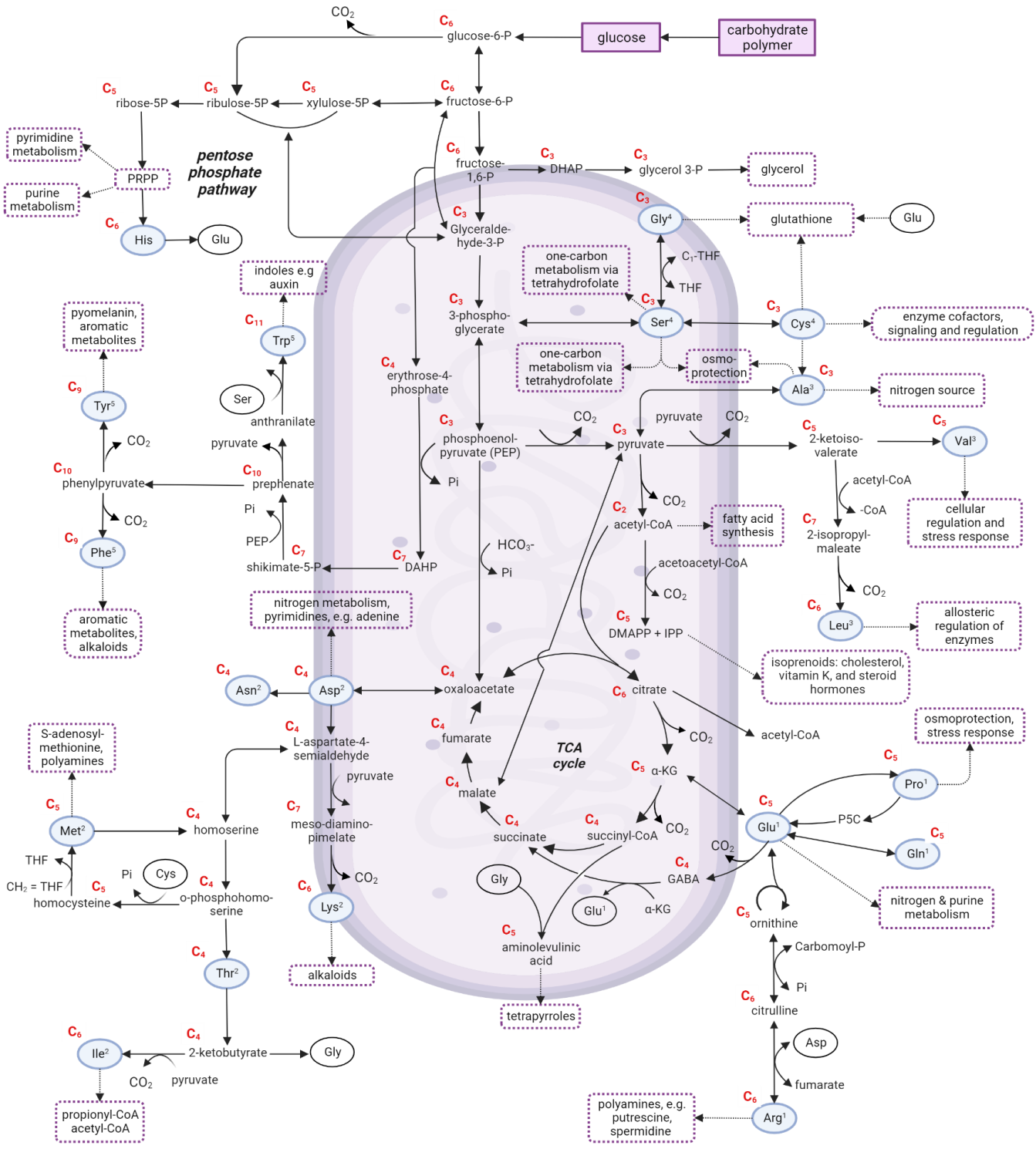

Figure S1B. Amino acid biosynthesis pathways in heterotrophic bacteria. Simplified schematic overview of the anabolic and catabolic amino acid (AA) pathways in heterotrophic bacteria using *Escherichia coli* as a model organism. The superscript number next to each AA indicates its categorization (see Fig. S1A) and the filled ellipses indicate that it is a product of the main biosynthesis pathway. The descriptions inside the rounded rectangles exemplify important non-proteinogenic functions of AAs in *E. coli*. Abbreviations: Ala, alanine; α-KG, alpha-ketoglutarate; Asn, asparagine; Asp, Asparagine; CBB, Calvin-Benson-Bassham; Cys, cysteine; DAHP, 3-deoxy-D-arabinoheptulosonate 7-phosphate; DMPP, dimethylallyl pyrophosphate; GABA, γ-Aminobutyric acid; Gly, glycine; Gln, glutamine; Glu, glutamic acid; His, histidine; Ile, isoleucine; IPP, Isopentenyl pyrophosphate, Leu, leucine, Lys, lysine; Met, methionine; P5C, 1-pyrroline-5-Carbo xylate; Phe, phenylalanine; Pro, proline; PRPP, Phosphoribosylpyrophosphate; RuBisCo, ribulose-1,5-bisphosphate carboxylase-oxygenase; Ser, serine; TCA, tricarboxylic acid; Trp, tryptophan; Tyr, tyrosine; Val, valine. The pathways are based on the KEGG PATHWAY database (https://www.kegg.jp/kegg/pathway.html). The illustration was created with BioRender.com.

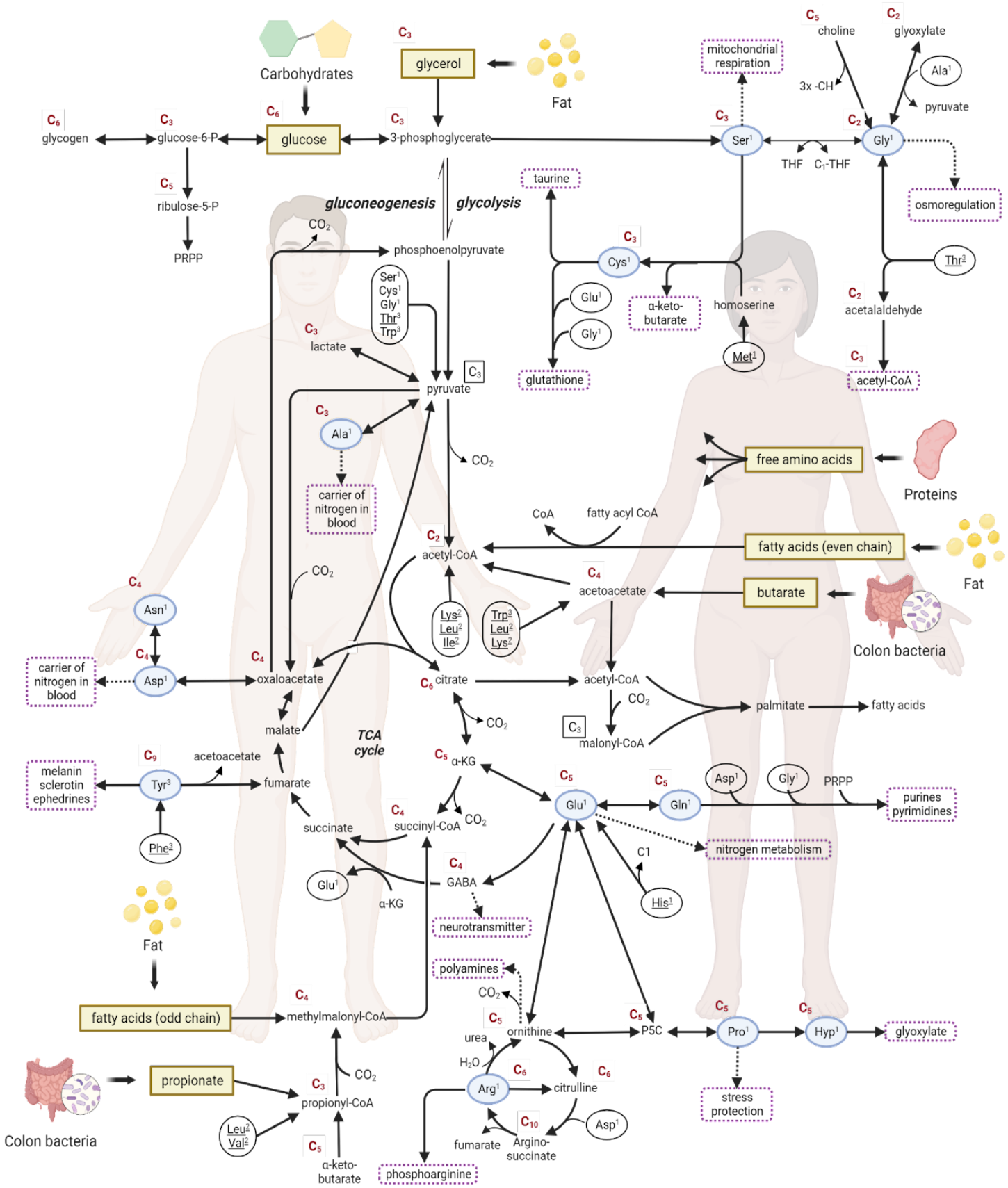

Figure S1C. Anabolic and catabolic AA pathways in vertebrates using *Homo sapiens* as a model organism. The non-essential AAs (filled ellipses) can be grouped according to their association with their main biosynthesis pathways: The glycolytic AAs are synthesised from metabolic intermediates (pyruvate, phosphoenolpyruvate) of the glycolytic pathway (in the cytosol) and the tricarboxylic acid (TCA) NEAAs are synthesised from intermediates of the TCA cycle (a-KG, oxaloacetate) (in the mitochondria). Glucose and glycerol are

sourced to the glycolytic pathway, and fatty acids (FAs) and short chain fatty acids are sourced to the TCA cycle. The catabolism of excess AAs either occurs via gluconeogenesis or ketogenesis. Gluconeogenesis is the synthesis of glucose from non-carbohydrate precursors such as the glucogenic AAs (marked with 1) and ketogenesis is the metabolic pathway for producing ketone bodies by breaking down fatty acids and ketogenic AAs (marked with 2). A large group of AAs can be catabolized by both processes (marked 3). Key roles of the non-essential AAs as precursors in physiological processes other than protein synthesis are indicated within the rounded rectangles. Certain non-proteinogenic AAs such as citrulline and ornithine are important intermediaries in various pathways involving nitrogenous metabolism. In terms of the macronutrients, carbohydrates primarily serve as an energy source after being converted to glucose and then to glycolytic intermediates such as 3-phosphoglycerate and pyruvate before entering the TCA cycle. If the supply of carbohydrates exceeds the cell's immediate energy demand, it is stored in the liver as glycogen or, with the help of insulin, converted into fatty acids, circulated to other parts of the body and stored as fat in adipose tissue. Some carbohydrates also become NEAA building blocks. Proteins get converted to AAs in the digestive system before entering the liver. If the AAs are not used to build proteins, they are either catabolised via gluconeogenesis or ketogenesis. Gluconeogenesis is the synthesis of glucose from non-carbohydrate precursors such as the glucogenic AAs. Ketogenesis is the metabolic pathway for producing ketone bodies by breaking down fatty acids and ketogenic AAs. A large group of AAs can be catabolized by both processes. Lipids are converted to glycerol, fatty acids and short chain fatty acids. They are able to create energy in a process called beta oxidation that produces acetyl-coA. Some acetyl-coA molecules are used for synthesis of structural and functional lipids, and others are used as an energy source in the TCA cycle. Like the other macronutrients, fatty acids can also be used as NEAA building blocks. The metabolic pathways are summarised based on Frayn and Evans (2016). Other abbreviations: Ala, alanine; Asn, asparagine; Asp, Asparagine; Cys, cysteine; GABA, γ-Aminobutyric acid; Gly, glycine; Gln, glutamine; Glu, glutamic acid; His, histidine; Ile, isoleucine; IPP, Isopentenyl pyrophosphate, Leu, leucine, Lys, lysine; Met, methionine; P5C, 1-pyrroline-5-Carboxylate; Phe, phenylalanine; Pro, proline; PRPP, Phosphoribosylpyrophosphate; Ser, serine; Trp, tryptophan; Tyr, tyrosine; Val, valine. The illustration was created with BioRender.com.

Appendix S2

The power and pitfalls of amino acid carbon stable isotopes for tracing origin and use of basal resources in food webs

Vane K., Cobain M.R.D., Larsen T.

Ecological Monographs

Literature compilation of basal resource δ^{13}C-EAA data

The overview of individual observations can be found in the Figshare data repository: DOI:10.6084/m9.figshare.22852355.v2

To compile published δ^{13}C-EAA values of basal resources, we conducted a Web of Science search. This search, which covers data published until the end of 2022 and used a combination of keywords such as 'amino acid', 'carbon isotopes', 'ecology'. Initially, we compiled all the δ^{13}C-EAA values in basal resources that were directly measured in the study, and also screened additional references therein. We included all studies with available δ^{13}C-EAA values, whether they were published online or obtained by request. All studies included in our compilation obtained their δ^{13}C-EAA values through measurements made with a Gas Chromatography-Isotope Ratio Mass Spectrometry (GC-IRMS) system. The measurements were obtained with different derivatization protocols and in different analytical facilities. However, we did not correct for interlaboratory differences due to the lack of international reference materials with known δ^{13}C values. As a result, some of the variation in δ^{13}C-EAA values among basal resources may be attributed to methodological and analytical differences.

For comparisons between studies, we limited basal resources to those that were measured for five EAAs: leucine, isoleucine, valine, phenylalanine, and threonine. Lysine was not measured in the majority of the studies. Measurements of basal resources that were based on composite samples, such as POM, microbial mats or zooplankton were omitted to ensure that only those basal resources that were directly measured without potential addition of other basal resource traces or detrital materials. This also allowed us to be more precise with the assignment of basal resources to particular groupings, from general groupings of plants, bacteria, and phytoplankton to subgroupings of C_3/C_4/CAM plants, freshwater/marine phytoplankton, diazotrophy in cyanobacteria, brown/red macrophytes, seagrass, and green macrophytes (represented only by *Ulva* sp. plus one measure of *Batophora* sp.). Sample taxonomy was standardised according to the GBIF backbone (the Global Biodiversity Information Facility, GBIF 2022).

The discrimination of the baseline δ^{13}C-EAA values in these basal resources were then visualised by using a linear discrimination analysis (LDA). LDAs were typically limited to only three basal resource groups providing maximal discrimination that can be observed in 2-dimensional plots. In order to estimate the overlap between groups, we calculated the Bayesian posterior distribution of the Bhattacharyya coefficients (BC, Bhattacharyya 1946) of pairwise groups. The BC is a general statistical measure that quantifies the degree of similarity between two multivariate distributions, ranging from 0 (completely dissimilar distributions, i.e. no overlap) to 1 (identical distributions, i.e. complete overlap), regardless of the dimensionality of the data. This makes it highly suitable for δ^{13}C-EAA data, where the dimensionality of the data can vary between studies depending on the number of AAs that can be measured. This means measures of overlap can be compared either between studies, or contrasted pre- or post- transformations of data (e.g. PCA or LDA dimensionality reduction). In order to estimate the posterior distributions of BC for each pair, and therefore the overlaps, we derived Bayesian posteriors for multivariate normal distributions of basal group δ^{13}C-EAA patterns (post LDA) using an MCMC approach with the "fitMVNdirect" function given in Skinner et al. (2019) with the default settings. This is a generalised, dimension-wise, approach analogous to that implemented in the commonly used SIBER package (Jackson et al. 2011). The BC was then calculated pairwise for each posterior draw using the "bhattacharyya.matrix" function from the fpc package (Hennig 2023). Analyses were conducted in R statistical software version 4.2.1 (R Core Team 2022).

Table S2.1. Posterior estimates of Bhattacharyya coefficients for group pairs plotted in Fig. 3 of the main manuscript. Q25 and Q75 represent the interquartile range.

ID	pairing	min	q25	median	q75	max
A	Algae vs Bacteria	0.488	0.598	0.631	0.668	0.806
A	Algae vs Fungi	0.773	0.861	0.882	0.900	0.961
A	Algae vs Plants	0.411	0.510	0.531	0.554	0.655
A	Bacteria vs Fungi	0.252	0.421	0.465	0.511	0.693
A	Bacteria vs Plants	0.107	0.201	0.232	0.268	0.447
A	Fungi vs Plants	0.201	0.310	0.343	0.374	0.502
B	Brown algae vs Green algae	0.32	0.47	0.515	0.56	0.725
B	Brown algae vs Red algae	0.17	0.308	0.349	0.391	0.61
B	Brown algae vs Seagrass	0.018	0.084	0.118	0.169	0.556
B	Green algae vs Red algae	0.446	0.635	0.678	0.723	0.885
B	Green algae vs Seagrass	0.002	0.032	0.058	0.101	0.587
B	Red algae vs Seagrass	0.047	0.184	0.232	0.294	0.674
C	C_3 vs C_4	0.72	0.885	0.911	0.936	0.989
C	C_3 vs CAM	0.144	0.28	0.32	0.366	0.614
C	C_4 vs CAM	0.045	0.193	0.248	0.315	0.582

Table S2.2. Posterior estimates of Bhattacharyya coefficients for group pairs plotted in Fig. S2. Q25 and Q75 represent the interquartile range.

Subplot	Pairing	min	q25	median	q75	max
A	Cyanobacteria_D vs Freshwater phytoplankton	0.119	0.333	0.399	0.463	0.709
A	Cyanobacteria_D vs Marine phytoplankton	0.041	0.179	0.243	0.316	0.603
A	Freshwater phytoplankton vs Marine phytoplankton	0.143	0.295	0.341	0.383	0.581
B	Freshwater phytoplankton vs Marine phytoplankton	0.168	0.292	0.332	0.374	0.586
B	Freshwater phytoplankton vs Seagrass	0.042	0.162	0.215	0.288	0.666
B	Marine phytoplankton vs Seagrass	0.02	0.073	0.102	0.143	0.48
C	Bacteria vs Fungi	0.231	0.356	0.396	0.439	0.607
C	Bacteria vs Phytoplankton	0.125	0.23	0.267	0.305	0.48
C	Fungi vs Phytoplankton	0.233	0.349	0.39	0.434	0.643
D	Bacteria vs Macrophytes	0.31	0.449	0.485	0.522	0.682
D	Bacteria vs Plants	0.026	0.068	0.087	0.108	0.244
D	Macrophytes vs Plants	0.255	0.361	0.385	0.411	0.51

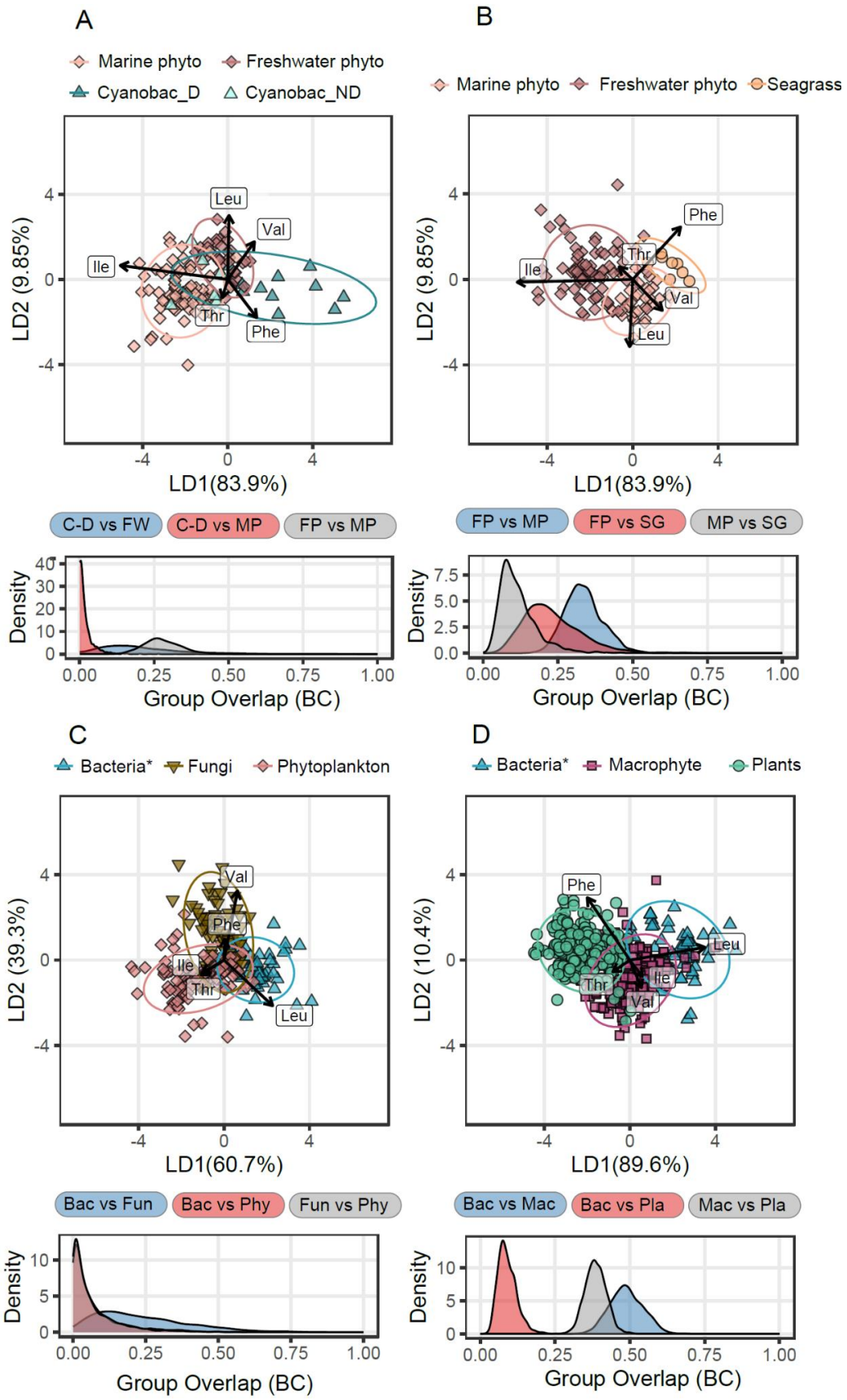
A
Marine phyto
Freshwater phyto
Cyanobac_D
Cyanobac_ND
LD2 (9.85%)
LD1(83.9%)
Leu
Val
Ile
Thr
Phe
C-D vs FW
C-D vs MP
FP vs MP
Density
Group Overlap (BC)
B
Marine phyto
Freshwater phyto
Seagrass
LD2 (9.85%)
LD1(83.9%)
Phe
Thr
Ile
Val
Leu
FP vs MP
FP vs SG
MP vs SG
Density
Group Overlap (BC)
C
Bacteria*
Fungi
Phytoplankton
LD2 (39.3%)
LD1(60.7%)
Val
Phe
Ile
Thr
Leu
Bac vs Fun
Bac vs Phy
Fun vs Phy
Density
Group Overlap (BC)
D
Bacteria*
Macrophyte
Plants
LD2 (10.4%)
LD1(89.6%)
Phe
Leu
Thr
Ile
Val
Bac vs Mac
Bac vs Pla
Mac vs Pla
Density
Group Overlap (BC)

Figure S2: Linear discriminant (LD) analysis of basal resources based on mean-centred δ^{13}C-EAA values compiled from the literature. Upper subplot panel: LD scores for individual samples, with distinct symbols denoting each group. Lower subplot panel: Bhattacharyya coefficients (BC) for group pairs represented as density scores, indicating the degree of overlap in LD scores between groups (0 = no overlap, 1 = identical distributions). EAAs considered: leucine (Leu), isoleucine (Ile), valine (Val), threonine (Thr), and phenylalanine (Phe). Each subplot features the following taxa: A) Freshwater phytoplankton, marine phytoplankton, diazotrophic cyanobacteria (Cyanobac_D), and non-diazotrophic cyanobacteria (Cyanobac_ND, predicted group); B) Bacteria, freshwater phytoplankton, and seagrasses; C) Bacteria, fungi, and phytoplankton; D) Bacteria, macrophytes (macroalgae and aquatic, and plants (comprising C_3, C_4, and CAM). For visual clarity, coefficients for each independent variable were multiplied by 8. See sample identities, classifications, and literature sources in Figshare DOI:10.6084/m9.figshare.22852355 and BC values in Table S2.

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Appendix S3

The power and pitfalls of amino acid carbon stable isotopes for tracing origin and use of basal resources in food webs

Vane K., Cobain M.R.D., Larsen T.

Ecological Monographs

Phylogeny contributes to variation in δ^{13}C-EAA patterns within vascular plants

As described in Section 2 of the main text, δ^{13}C-EAA patterns are expected to vary with phylogeny due to lineage specific biosynthetic pathways and enzymatic constraints. In addition there are confounding phenotypic expressions observed within taxonomic clades, i.e. clades are on average adapted to live in particular environments, that may also potentially influence δ^{13}C-EAA patterns through phenotypic expressions that modulate EAA demands, and therefore isotopic fractionations. Although distinctions are apparent across broad taxonomic clades (Section 3), current data are too limited to test widely across basal organisms whether finer scale distinctions are readily quantifiable, although it is suggested in some specific instances (e.g. separation between cultured diatom species, Vane et al. 2023). Here we show however that phylogeny, within the relatively well described phylum of Tracheophyta (vascular plants), explains considerable variation in individual δ^{13}C-EAA patterns.

We limited our compilation dataset (in Appendix S2) to vascular plants only. As family was the lowest common taxonomic rank identified across all samples, we defined phylogeny from Tracheophyta down to family for each sample. Initial taxonomic ranks were extracted from GBIF (the Global Biodiversity Information Facility, GBIF 2022). To ensure that residual variation in δ^{13}C-EAA patterns could be adequately estimated, we further restricted the dataset to only those families with at least 3 observations, resulting in 18 families in total. Family names were cross referenced against the Open Tree of Life (OTL) and any families that had broken phylogenies (e.g. are paraphyletic) were reassigned to monophyletic subfamily divisions that incorporated all samples from the original family. The phylogenetic subtree of these 18 (sub)families was then extracted from OTL (shown in Fig. S3A, using the R package 'rotl', Michonneau et al. 2016).

To test whether phylogeny explains variation in δ^{13}C-EAA patterns, we constructed a multivariate, phylogenetic mixed effects model in a Bayesian framework, with the five mean-centred EAA δ^{13}C values modelled as a response to the random effect of phylogenetic relatedness based on the topology of the vascular plant family subtree. The model was run in R (version 4.2.1, R Core Team 2022) using the package 'MCMCglmm' (Hadfield, 2010, model specifics are provided in the supplied R code). Trace plots of the chain were checked and showed good model convergence.

The average δ^{13}C-EAA pattern for a vascular plant is shown in Fig. S3A. Mean-centred threonine values are relatively enriched in ^{13}C (median 12.1‰) and leucine relatively deplete (median -8.2‰) compared to other EAAs. However, these two EAAs also had the least certainty of their means, shown by the wide credibility intervals (CIs), followed by isoleucine. Average phenylalanine and valine had much higher certainty, with 95% credibility intervals spanning <1‰. Approximately 50% of the total variation in δ^{13}C-EAA patterns was attributed to phylogeny (posterior median = 0.51, 95% CI 0.36 to 0.66). Mean-centred leucine δ^{13}C values varied the most with phylogeny (mean variance of 9.7, 95% CI 3.0 to 18.5), followed by threonine (mean 5.0, 95% CI 0.44 to 11.4) and isoleucine (mean 3.1, 95% CI 0.62 to 6.8), with almost no variation expressed in either phenylalanine (mean 0.15, 95% CI <0.01 to 0.65) or valine (mean 0.31, 95% <0.01 to 1.6), shown in Fig. S3A. Qualitatively, it can be observed that some families express similar δ^{13}C-EAA patterns despite being phylogenetically distant from each other. Notably, Fabaceae, the legume plants that typically host nitrogen fixing bacteria in their roots, have relatively deplete threonine values but relatively enriched leucine values. This matches with the distant sister families Posidoniaceae and Hydrocharitaceae, which encompass marine seagrasses and many other aquatic plant species. It can also

be seen that the families Agavoideae, Cactaceae and Zygophyllaceae have similar δ^{13}C-EAA patterns marked by relatively deplete leucine but enriched isoleucine and threonine, with representative species typically known for being adapted to dry habitats.

The residual variance - covariance structure is shown in Fig. S3B. Threonine expressed the largest individual variation (mean variance 7.35) and negatively co-varied with all other EAAs (all mean correlations < -0.39). These negative correlations intuitively make sense as threonine is the most relatively enriched AA (Fig. S3B) and the data are mean centred, therefore increasing values in one AA will be accompanied by decreases in the other EAAs. Interestingly, valine and phenylalanine, despite showing almost no variation with phylogeny, express considerable individual variances (means of 3.05 and 3.97 respectively). This implies that mechanisms at the individual level result in variation in these EAAs rather than lineage specific mechanisms. Valine and phenylalanine also negatively covary with each other, likely due to the same reasoning as with threonine. Despite having large variations with phylogeny, isoleucine and leucine both have limited individual variances, suggesting that within vascular plants, metabolic networks involving these two AAs may be less plastic at the individual level.

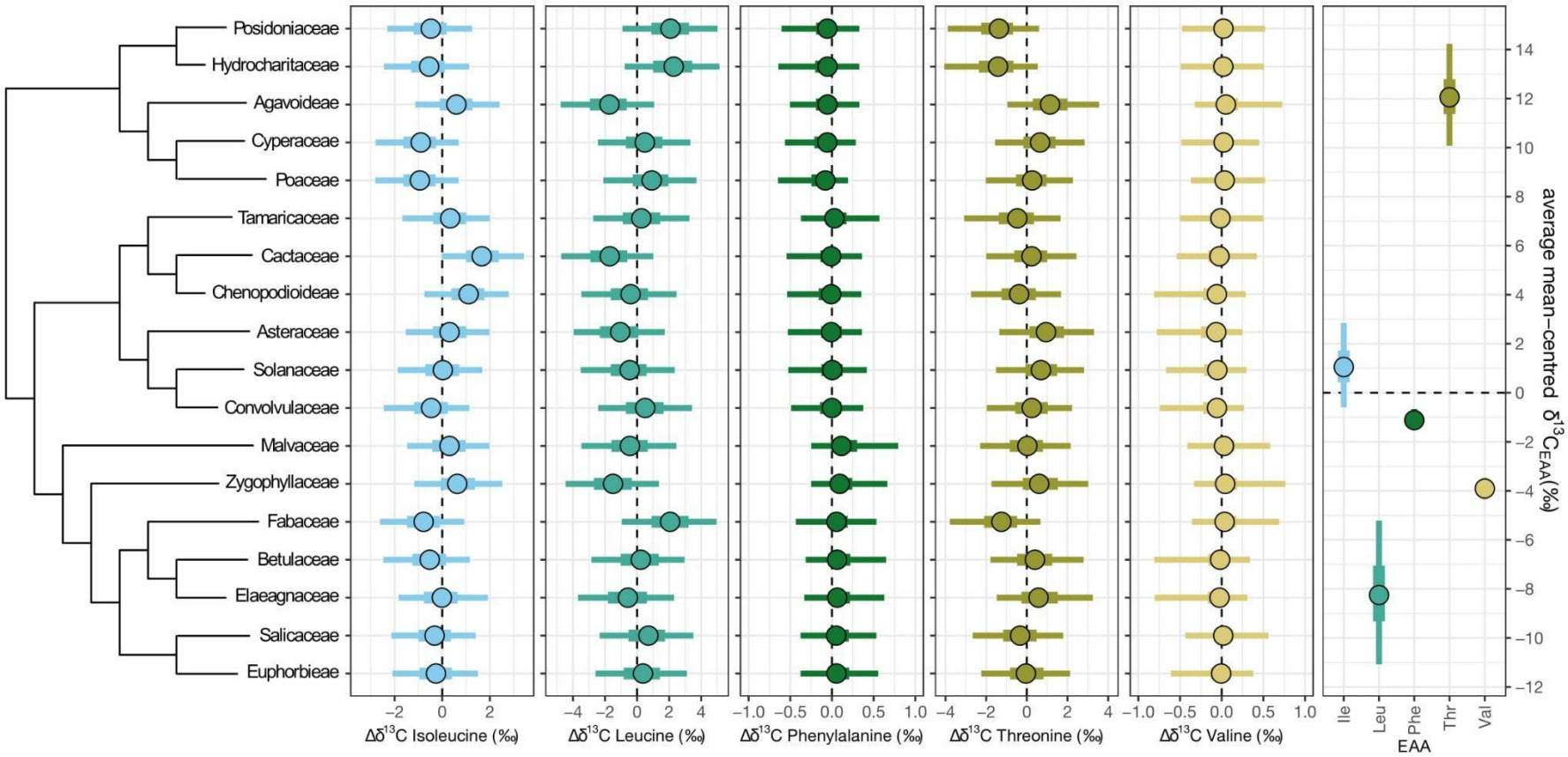

Figure S3A: Modelled mean-centred $\delta^{13}C$ values of five EAAs ($\delta^{13}C$ patterns) of vascular plants. Global average values (right hand panel) and the offsets, $\Delta\delta^{13}C$, for each EAA (first to fifth panels) among the 18 taxonomic (sub)families in the vascular plant dataset. Phylogenetic topology between the 18 families is plotted on the left hand side. Circles indicate median posterior values, thick bars denote the 50% credible intervals (CIs) and thin bars the 95% CIs. Average mean-centred $\delta^{13}C$ CIs for phenylalanine and valine fall within the median circles. Dashed lines are plotted at zero on all panels for clarity. This figure is an enlarged version of the subplot Figure 4D from the main manuscript.

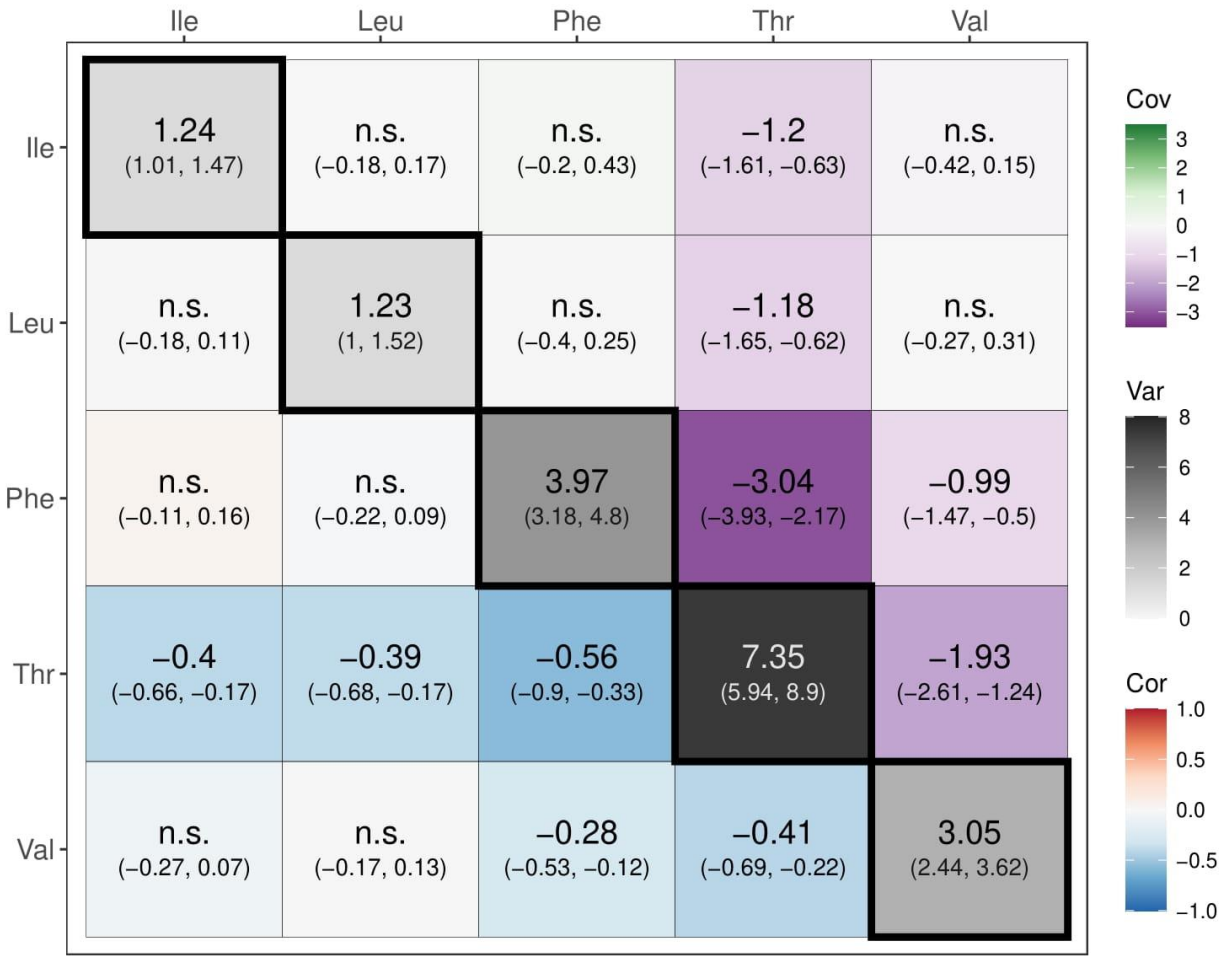

Figure S3B: Residual variance - covariance matrix of modelled vascular plant $\delta^{13}C$ patterns. Variances (Var) of individual EAAs are plotted along the diagonal with thick borders, covariances (Cov) in the upper triangle, and corresponding correlations (Cor) in the lower triangle. Posterior mean values (large text) with 95% credible intervals (smaller text) are given for each EAA pairing. Posterior mean values that are not statistically distinguishable from zero are denoted as n.s.

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Appendix S4

The power and pitfalls of amino acid carbon stable isotopes for tracing origin and use of basal resources in food webs

Vane K., Cobain M.R.D., Larsen T.

Ecological Monographs

Literature compilation of archaeological human δ^{13}C-AA data

The overview of selected studies and individual samples can be found in the Figshare data repository: DOI:10.6084/m9.figshare.22852355.v2

We compiled δ^{13}C-AA data from historical and archaeological human populations with diverse subsistence strategies, as reported in eight studies. In two of these studies (Honch et al. 2012 and Colonese et al. 2014), the archaeological and environmental contexts enabled us to select a subset of populations for which we could identify their primary dietary protein sources: freshwater (δ^{13}C-EAA mean: -27.5±1.5‰, n=12), marine (-19.4±1.2‰, n=19), terrestrial C_3 (-27.1±1.0‰, n=12), and terrestrial C4 (-19.1±2.1‰, n=14) proteins. The protein sources for the six populations from the remaining six studies were less certain. These populations included individuals from Köpingsvik (bone, Mesolithic and Middle Neolithic; Webb et al. 2018); Nancheng (bone, Proto-Shang; Ma et al. 2021); Nukdo (bone, Late Bronze Age; Choy et al. 2010); Odense rib (bone, Medieval; Brozou et al. 2022); Odense femur (bone, Medieval; Brozou et al. 2022); Pica-8 (hair, Late Intermediate; Mora et al. 2018); Pica-8 (tendon, Late Intermediate; Mora et al. 2018); and Uummannaq (bone, 16th and 17th centuries; Raghavan et al. 2010). Two of the studies reported δ^{13}C-AA values for different tissue types from the same individuals. We compared two types of data preprocessing: measured and EAA mean-centred δ^{13}C-AA data. The former highlights the influence of environmental factors on δ^{13}C-AA variations, while the latter emphasises the effect of metabolic processes on intermolecular δ^{13}C variability. We applied two ordination techniques, PCA and LDA, to assess the relationship between the independent variables (i.e., δ^{13}C-AA values) and the spread of data within and between groups with known primary diet protein sources. We then projected the δ^{13}C-AA values of individuals with unknown protein sources onto the principal component and linear discriminant spaces. To corroborate the correctness of the results, we used mean δ^{13}C-EAA values (phenylalanine and valine), with marine and C_4 protein groups expected to be more ^{13}C enriched than the freshwater and C_3 protein groups. We employed two different methods to assess the similarity of humans to the four protein sources:

1) For both preprocessing datasets, we compared class probability assignments $p\theta(x)$ and likelihood $lx(\theta)$ functions to predict protein sources in the LDA output. While $p\theta(x)$ is best suited for discrete classification to a predefined group because it sums to 1, $lx(\theta)$ is not normalised to 1 and is therefore less prone to false inferences by forcing unlikely classifications.

2) To measure the similarity of the populations to the protein groups across both preprocessing datasets and ordination methods, we calculated Bhattacharyya coefficients, which measure similarity between two multivariate probability distributions (see Appendix S2 for statistical details). A coefficient of 0 indicates no overlap between the two distributions, while a coefficient of 1 indicates that they are identical.

PCA captures the direction of maximum variation in the data rather than maximising group separability as is the case for LDA. Therefore, variables contributing to intragroup variation have a greater weight in PCA than LDA. This is particularly apparent when separating the C_3 and freshwater protein groups from the C_4 and marine protein groups based on baseline δ^{13}C-EAA values. In terms of classifying new observations (individuals with unknown protein source), LDA will assign them to the class with the highest likelihood, even if it is small. If the highest likelihood is small, the observation has weak similarities to any of the predefined classes. The Pica 8 hair individuals exemplify this, as the δ^{13}C values of glycine are enriched by ~10‰ compared to glycine in the collagenous Pica 8 samples. This shows that LDA predictions can be misleading when the training data are inadequate or fall outside the boundaries of the training data. Identifying these observations can be achieved through visual inspection of discriminant scores and

likelihood estimates. From visual inspection, it is evident that the Pica-8 hair samples fall outside the predefined C_4 protein group and have much lower likelihoods compared to the tendon samples - see Figs. 6B1 in the main manuscript and Fig. S4, specific values provided in the metadata overview on Figshare Data Repository linked above. In contrast to likelihood estimates, which provide values for single observations, Bhattacharyya coefficients (BC) are estimated at the population level, for two compared groups, in this case, a human population versus a protein source. The median BC values of Bayesian posterior estimates reported in Fig. S4 show that PCA ordination generally produces higher median values compared to LDA ordination. This is to be expected as LDA optimises for separation between groups. However, these median BC values have limited value for this case study because populations falling within the 'mixing-space' of the four protein sources but not overlapping with any of them have low BC values. Therefore, it is important to visually inspect the ordination plots when evaluating BC values. Nevertheless, many of the trends reported in likelihood estimates also hold true for the BC values. For instance, both the Odense (both rib and femur) and Nukdo populations exhibit a much greater overlap with C_3 proteins in the PCA than in the LDA techniques, underscoring how sensitive these predictions are to the specific ordination method used.

Out of 64 unknown (predicted) individuals, 18 were categorised differently between the two data set representations (measured vs. mean-centred) due to the slight structural differences. According to the LDA output, most of these individuals likely consumed mixed diets, e.g. Nukdo individuals on C3/marine protein (δ^{13}C-EAA mean: -25.9±1.1‰, n=9), or on brackish resources, e.g. the Köpingsvik (-22.1±0.5‰, n=5) and Uummannaq (-20.4±0.5‰, n=6) individuals. The measured values for the Uummannaq individuals have a marine protein bias, while the mean-centred values have a freshwater protein bias (Fig. 6 in the main manuscript). For the Odense individuals (femur: -26.3±0.7‰, n=10; rib: -26.2±1.2‰, n=10), the measured values categorised all but one femur sample as C3, while the mean-centred data categorized 11 in C_3, 3 in marine, and 6 in C_4 group. The mean δ^{13}C-EAA values and contextual information support the predictions based on measured values for the Uummannaq individuals and non-C_3 predictions of the Odense individuals. The prediction of individuals from the remaining populations (Nancheng, -15.4±2.2‰, n=12; Pica 8 tendon, -19.3±3.8‰, n=6; Pica 8 hair, -16.4±1.3‰, n=6) are consistent between the two data sets with all but one individual clustering with the C4 protein group. The prediction of the outlier individual (SE-T3) with the C_3 protein group is corroborated by its mean δ^{13}C-EAA value (-26.8‰). A visual inspection shows that predictions based on measured δ^{13}C-EAA values are more accurate, as seen in Figs. $6B_1$ and $6D_1$. For example, the individual (M70) with a mean δ^{13}C-EAA value of -22.1‰ is a clear outlier in Fig. $6B_1$, trending towards the C_3 group, while a similar trend is less obvious in Fig. $6D_1$.

The data compilation comprises two populations, Pica-8 and Odense, from which it is possible to infer dietary histories from the same individuals thanks to analyses of different tissue types. The earlier dietary history of the Pica-8 individuals represented by the tendon samples indicates that the individuals relied on different subsistence strategies: Terrestrial C_4 (n=4), marine C_4 (SI-T74; n=1), and possibly a mixture of terrestrial C_3 and C_4 (SE-T3; n=1). The comparatively higher mean δ^{13}C-EAA values of the hair than tendon samples, typically between 1 and 2‰, support that the population became more reliant on C_4 protein sources. This is particularly true for the SE-T3 whose hair samples were ^{13}C enriched by 8.6‰ compared to the tendon samples, which had a mean δ^{13}C-EAA value typical of the terrestrial protein group. We are

also questioning whether the classification of the SI-T74 and SE-T3 tendon samples to the C_4 group is correct in part because of their mean δ^{13}C-EAA values are depleted by ~3‰ compared to the the remaining Pica-8 individuals and most of the C_4 Nancheng individuals. Thus, it appears that the ordination and mean δ^{13}C-EAA results do not fully corroborate one another. In terms of inferring dietary histories based on collagen only, the ribs of the Odense individuals most likely represent the period after they were admitted to a leprosy hospital and the femurs represent earlier periods. As noted by the authors of the study, it appears that several individuals increased marine protein consumption after hospitalisation (Brozou et al. 2022). For individuals relying on proteins from brackish waters, the predictions of the Uummannaq (Raghavan et al. 2010) and Köpingsvik (Webb et al. 2018) populations are in line with modern salinity observations showing brackish waters in both locations, but with the protein sources of the Uummannaq individuals being more marine-based compared to the Köpingsvik individuals (Holinde and Zielinski 2016, Kniebusch et al. 2019). Most Nukdo individuals relied more on marine than C_3 proteins (Choy et al. 2010).

Regardless of the preprocessing and ordination methods, both datasets have many similar features in terms of the weight and direction of independent variables (Fig. 6B2 vs. Fig. 6D2): Alanine, aspartate, and glutamate generally contribute to maximising intragroup variation (Fig. 6C2), and phenylalanine, valine, proline, and glycine contribute to maximising intergroup variation (Fig. 6B2 and 6D2). Our study confirmed that δ^{13}C of phenylalanine vs. valine separate terrestrial and aquatic resources (Honch et al. 2012, Larsen et al. 2013). Like previous studies, we found that phenylalanine relative to valine is more ^{13}C enriched in terrestrial than in aquatic protein groups. Among the NEAAs, proline is important for separating the C_3 from the other protein groups. Our analysis could not determine the cause of the ^{13}C enrichment in the C_3 protein group compared to other groups. However, according to Liu et al. (2018), copepods on a high-carbohydrate diet exhibited a higher trophic ^{13}C enrichment of proline than anchovies on a high-protein diet. The ^{13}C enrichment of glycine is highest in the freshwater protein group and lowest in the C_4 protein group (Fig. 6B2). The cause of these isotopic effects remains unclear, as they could result from either metabolic processes in the food sources or post-ingestive processes. Factors contributing to these effects may include the conversion of excess dietary protein into fat and energy, as well as the de novo synthesis of glycine. Although alanine and glutamate are relatively uninformative AAs, the terrestrial protein groups were significantly more ^{13}C enriched than the aquatic protein groups ($P < 0.001$). This difference may arise from higher carbohydrate consumption in terrestrial protein groups compared to aquatic protein groups. Epidemiological studies investigating the δ^{13}C-AA response to high-fructose corn syrup-sweetened beverage intake have identified alanine and glutamate as potential markers of carbohydrate intake (Choy et al. 2013, Yun et al. 2018, 2020, Johnson et al. 2021). Both NEAAs use pyruvate, a glycolytic intermediate, as a precursor, and acetyl-CoA, a product of beta-oxidation, acts as a precursor for glutamate but not glycine (see Appendix S1, Fig. S1C). The distinct response of alanine and glutamate to carbohydrate intake is likely influenced by the balance of dietary fat to carbohydrate.

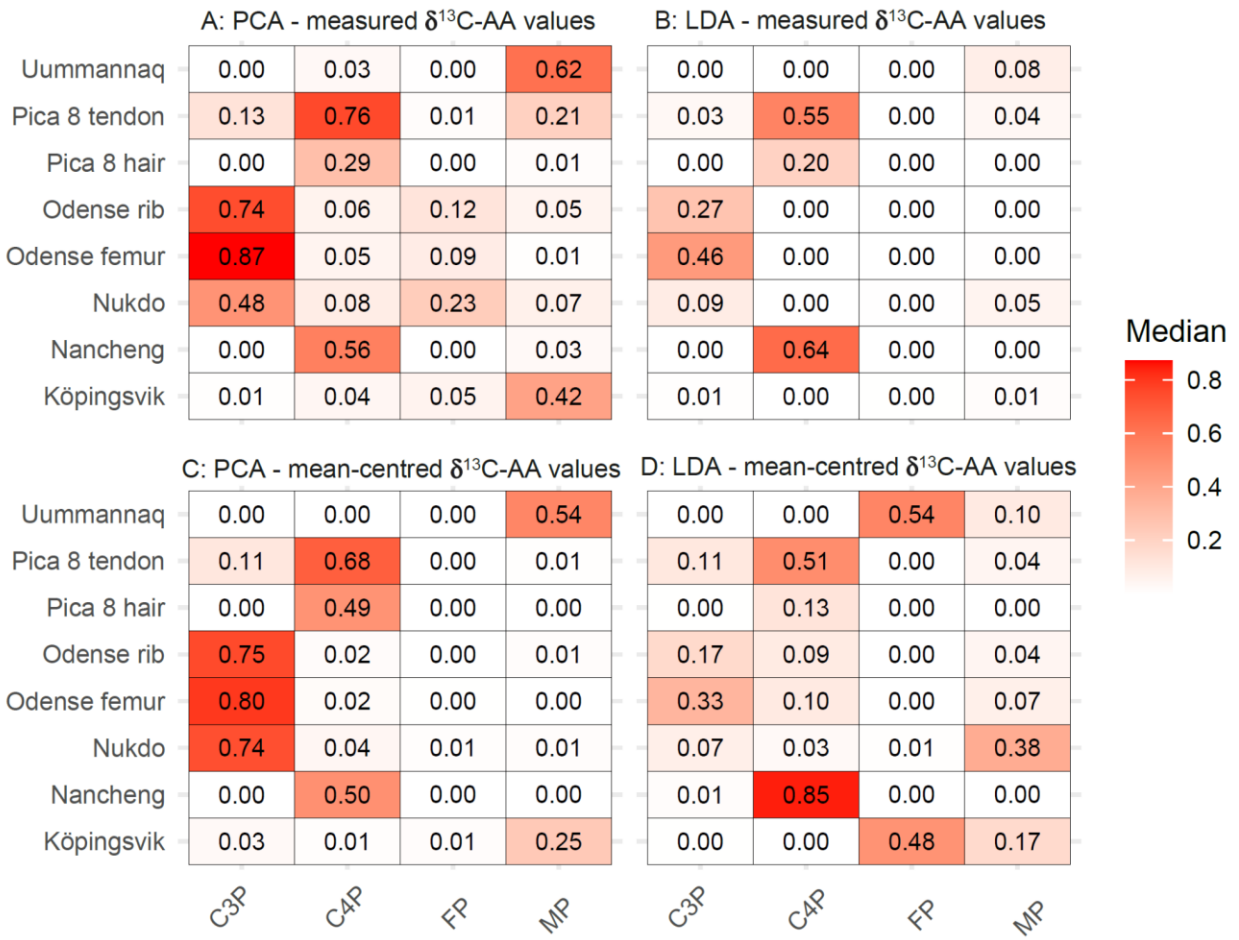

Figure S4: Comparison of human populations of known diets with those with uncertain diets. The matrix plots show the median Bhattacharyya coefficients (0 = no overlap, 1 = identical distributions) indicating the degree of overlap in PC (left hand side) or LD (right hand side) scores between human groups (see Fig. 6 in main manuscript) and their potential dietary protein sources (FP, freshwater protein; MP, marine protein, C3P, terrestrial C_3 protein; C4P, terrestrial C_4 protein). Top panels are based on measured δ^{13}C-AA data whereas bottom panels are EAA (phenylalanine and valine) mean-centred δ^{13}C-AA values.

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Appendix S5

The power and pitfalls of amino acid carbon stable isotopes for tracing origin and use of basal resources in food webs

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Ecological Monographs

The compilation of dietary offsets between $\delta^{13}C_{EAA}$ values in diet and metazoan tissues

The methodology overview of each study can be found in the Figshare data repository: DOI:10.6084/m9.figshare.22852355.v2

To investigate the differences in offsets between δ^{13}C-EAA values between diet and metazoan tissues, δ^{13}C-EAA values from controlled feeding studies that aimed to study the routing of AAs from different dietary compositions to animal tissues with δ^{13}C-AA values were compiled. We searched Web of Science for articles published from the beginning of online records until May 2022 using the terms “amino acid”, “carbon isotopes”, “fractionation”. While not all feeding experiments aiming to qualify the routing of AAs with carbon isotopes could be found with these search terms, references within publications mentioning trophic discrimination of EAAs were additionally screened.

As a result, 17 publications were found that described the measured δ^{13}C-EAA values between animal tissue and their specific diet. Analytical and methodological information was extracted from each publication and compiled. Extracted analytical information included instrumentation (e.g. GC-IRMS or LC-IRMS) derivatisation method in case of GC-IRMS measurements, description of the chemical pretreatments of both consumer and diet tissues. Methodology descriptions encompassed consumer species, type of tissue, dietary type or variations, amount of days that the diet was fed to the consumer, and how many individual replicate consumer tissues were measured. δ^{13}C-EAA values were mainly acquired from tables in the publication, online supplementary materials and data repositories, or direct requests to the corresponding author. However, offsets in δ^{13}C-EAA values were gained from a graph in Howland et al. (2003) as the request for raw data was unanswered. No corrections to the δ^{13}C-EAA values between the studies were necessary due to the interest in the offsets in δ^{13}C-EAA values between diet and tissue that were measured in the same analytical facility. As not all offsets were presented in a similar manner between publications (e.g. $\delta^{13}C\text{-}EAA_{tissue}$ - $\delta^{13}C\text{-}EAA_{diet}$), some offsets were calculated directly from study data.

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