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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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Abstract

Natural and anthropogenic stressors alter the composition, biomass, and nutritional quality of primary producers and microorganisms, the basal organisms that synthesize 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, for the first time, conceptualizes these mechanistic underpinnings that drive δ^{13}C-EAA fingerprints among basal organisms and incorporates 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 hypothesized AA-specific isotope fractionations among basal organism clades, demonstrating that phenylalanine separates vascular plant δ^{13}C-EAA fingerprints, which strongly covaries 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 scrutinizing the many methodologies that are applied in the field, we highlight the absence of standardized 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.

KEYWORDS
biotracer, fingerprints, food web tracing, microbes, patterns, review, spatiotemporal

INTRODUCTION

Food webs are increasingly impacted by anthropogenic stressors such as accelerated climate change, biodiversity loss, habitat destruction, and pollution (Blanchard et al., 2012; Hoegh-Guldberg & Bruno, 2010; 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—synthesized 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 (Kortsch et al., 2015; Nakazawa, 2015; Svanbäck et al., 2015). However, changes in basal organisms and the assimilation of their basal resources by higher trophic levels occur across spatiotemporal scales and on fine-scale taxonomic levels (Chidawanyika et al., 2019; McMeans et al., 2015; Raubenheimer et al., 2012). 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 quantifying the pathways of energy and nutrients in food webs. The relative abundance of heavy (^{13}C) to light (^{12}C) carbon isotopes, normalized to the international standard (Vienna Pee Dee Belemnite, VPDB) and expressed as $\delta^{13}C$ per mille (‰) values, are measured within whole organisms or bulk tissues. The $\delta^{13}C$ values of consumer tissues are then compared with their potential resources. $\delta^{13}C$ values are highly suited to trace basal resources because carbon is abundant, ubiquitous, and $\delta^{13}C$ values of basal organisms are often habitat or taxon specific. However, bulk $\delta^{13}C$ values of basal organisms can vary substantially with the environment (Casey & Post, 2011; Magozzi et al., 2017; Peterson & Fry, 1987), 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 organisms in a given ecosystem and contributions from microorganisms are frequently underappreciated due to the logistical challenge of sampling them in situ (Casey & Post, 2011).

To address the constraints of bulk tissue analysis, researchers increasingly analyze $\delta^{13}C$ values of individual biomolecules (Nielsen et al., 2017; Ruess & Müller-Navarra, 2019). Basal organisms take up external carbon to synthesize their own complex biomolecules. Following ingestion, digestion, and absorption, these biomolecules are assimilated into consumer tissues with minimal modification of their carbon skeleton, 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 in identifying specific basal organisms from primary producers and microbial organisms. $\delta^{13}C$-AA values can trace the carbon transfer from basal organisms to higher trophic levels, irrespective of environmental conditions (Elliott Smith et al., 2022; Larsen et al., 2009; Vane et al., 2023), serving as powerful spatiotemporal tracers of basal resource use. As AAs exhibit stable preservation in fossilized biogenic carbonates such as dinosaur eggshells, coral skeletons, and fish otoliths or other preserved structural tissues (Abelson, 1954; Hare et al., 1991; Ma et al., 2021; Mora et al., 2018), $\delta^{13}C$-AA values allow for detailed retrospective inferences of basal resource use by animals across contemporary, paleontological, and geological records.

Animals cannot synthesize 9 proteinogenic AAs de novo. While the non-synthesizable AAs, or essential amino acids (EAAs, Wu et al., 2014), must primarily come from dietary sources, gut microbes can synthesize them from various precursors including carbohydrates and NEAAs (Figure 1). In animals with adequate protein intake, microbial contribution to EAA requirements appears limited (Bergen, 2015). These contributions can become substantial in consumers specialized on ligneous, protein-poor diets (e.g., Ayayee et al., 2016; Larsen, Ventura, et al., 2016). In well-nourished animals that do not rely on coprophagy (Torrallardona et al., 2003), EAAs are predominantly routed from dietary proteins to tissues, resulting in negligible tissue-diet $\delta^{13}C$ offsets

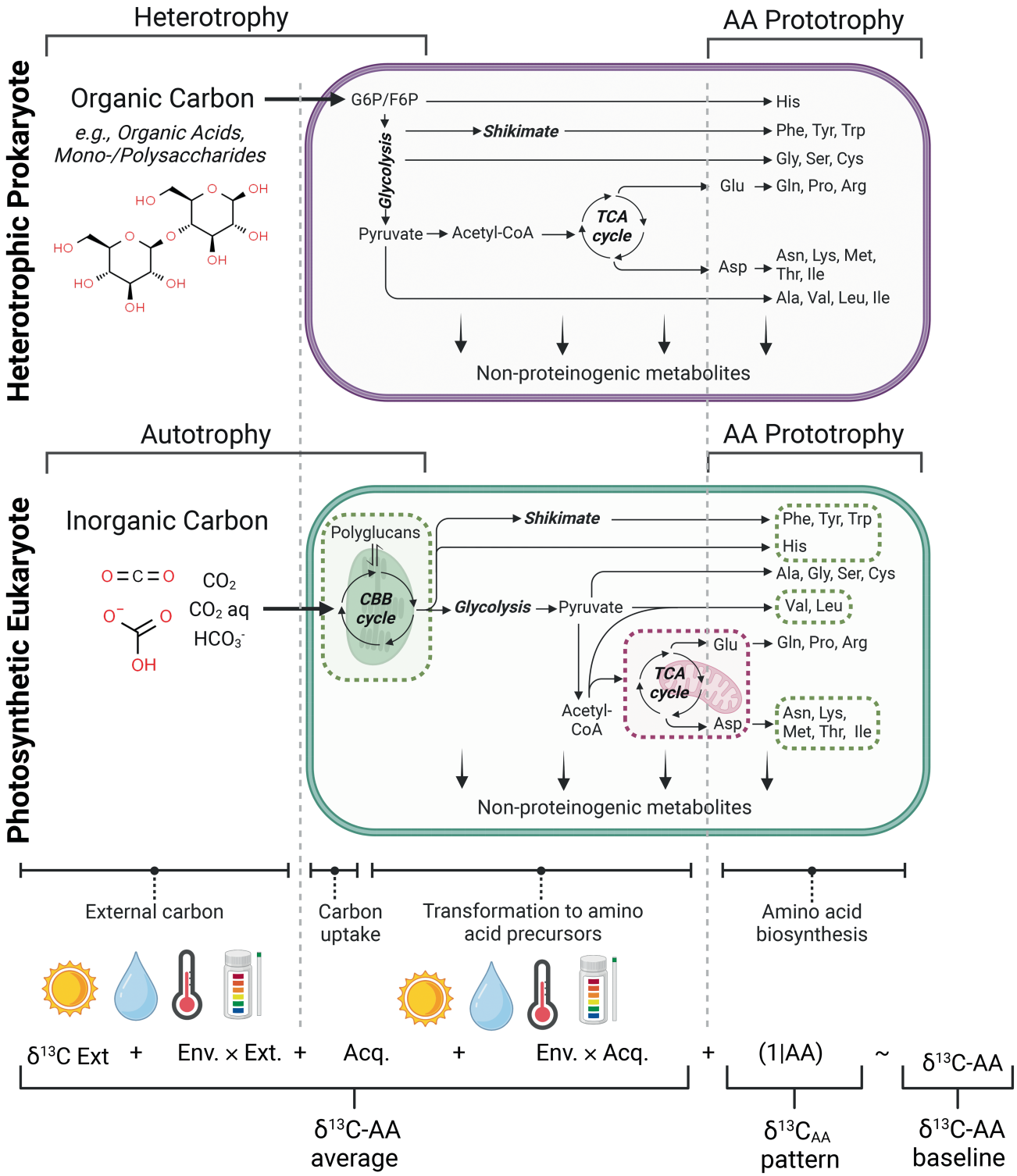

FIGURE 1 Schematic representation of the sources, processes, and environmental effects that contribute to the $\delta^{13}C$ values of synthesized proteinogenic AAs (Ala, alanine; Arg, arginine, Asn, asparagine; Asp, Asparagine; Cys, cysteine; Gly, glycine; Gln, glutamine; Glu, glutamic acid; His, histidine; Ile, isoleucine; Leu. leucine, Lys, lysine; Met, methionine; Phe, phenylalanine; Pro, proline; Ser, serine; Thr, threonine, Trp, tryptophan; Tyr, tyrosine; Val, valine) in two prototrophs—a heterotrophic prokaryote and a photosynthetic eukaryote, following Equations (1)–(3) in section *Conceptualizing amino acid $\delta^{13}C$ values in basal organisms*. 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 S1 and S2. Acetyl-CoA, acetyl coenzyme A; Acq., carbon acquisition; CBB, Calvin–Benson–Bassham; Env., environment; Ext., external; F6P, fructose-6 phosphate; G6P, glucose-6 phosphate; TCA, tricarboxylic acid. The illustration was created with BioRender.com.

(McMahon et al., 2010; McMahon, Polito, et al., 2015; Takizawa et al., 2017; Wang, Wan, et al., 2019). 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 δ^{13}C-EAA patterns: the relative differences in δ^{13}C values between EAAs (Elliott Smith et al., 2018, 2022; Larsen et al., 2013, 2015; Lynch et al., 2016; Scott et al., 2006; Stahl et al., 2023). Distinct δ^{13}C-EAA patterns among basal organisms that remain largely consistent across variable physiochemical conditions and through time have been typically referred to as δ^{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, some NEAAs can be considered conditionally essential for metazoans, particularly during stages of rapid growth when the rate of utilization outpaces the rate of synthesis, constraining normal physiological and metabolic processes without dietary supplementation (Eisert, 2011; Hou et al., 2015; Tresia et al., 2023; Wu, 2009).

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 fingerprint approach is limited (Besser et al., 2022; Nielsen et al., 2017; Ruess & Müller-Navarra, 2019; Whiteman et al., 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, Polito, et al., 2015). 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 standardized 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. Emphasizing 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.

FACTORS SHAPING AMINO ACID δ^{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 δ^{13}C-AA patterns in basal organisms vary across the diversity of life. The rigorous application of δ^{13}C-AA patterns, therefore, requires the development of a mechanistic framework (Hayes, 2001), which has so far been lacking. While variations between different biosynthetic pathways have been acknowledged as a key driver in diverging δ^{13}C-AA patterns among taxa (Larsen et al., 2009), ecological applications of δ^{13}C-AA patterns are still mostly driven by phenomenological observations (e.g., Besser et al., 2022; Larsen et al., 2012; Stahl et al., 2023). By conceptualizing the processes that give rise to δ^{13}C-AA values in basal organisms, we highlight how specific mechanisms can dominate the relative δ^{13}C offsets of certain AAs, underpinning the distinction of δ^{13}C-AA patterns between taxa. Explicit definitions for δ^{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 δ^{13}C-AA in ecological research.

Conceptualizing amino acid δ^{13}C values in basal organisms

Basal organisms are those that can synthesize basal resources de novo, here specifically considered the full suite of 20 proteinogenic AAs. The ability to synthesize particular biomolecules, such as AAs, is termed prototrophy (the inability being auxotrophy). The majority of basal organisms, the AA prototrophs, by biomass 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 break

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 synthesized 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 synthesized 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 synthesize particular biomolecules de novo (applied here specifically to EAA synthesis, antonym of prototrophs).
Basal organisms	Primary producers and microbes that synthesize suites of biomolecules de novo from externally sourced (in)organic carbon, considered to be the base of food webs.
Basal resources	The suites of biomolecules (focusing on AAs in this review) synthesized de novo by basal organisms and assimilated by consumers for normal physiological functioning.
Basal resource use reconstruction	Estimating the proportions of basal resources synthesized by specific basal organism groups or clades that have been assimilated into consumer tissues.
Facultative EAA-prototrophs	Organisms that can synthesize EAAs de novo, but have the capacity to assimilate externally derived EAAs for normal metabolic functioning.
Obligate EAA-prototrophs	Autotrophs that synthesize all 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 characterize basal organisms in another 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 standardized to VPDB.
Measured δ^{13}C-AA values	The VPDB standardized (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 multivariate δ^{13}C-EAA pattern space that is solely occupied by a group or collection of similar basal organisms, encompassing the intragroup variability in δ^{13}C-EAA patterns expressed by those organisms.

down organic molecules into simple 2- or 3-carbon compounds for both chemical energy and de novo synthesis of biomolecules (Hayes, 2001). The pathways from external sources of (in)organic carbon to intracellular AA synthesis can be generalized 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 synthesize 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, Fry, 2006; Hayes, 2001). The carbon isotope composition of individual AAs 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 S1 and S2). 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.

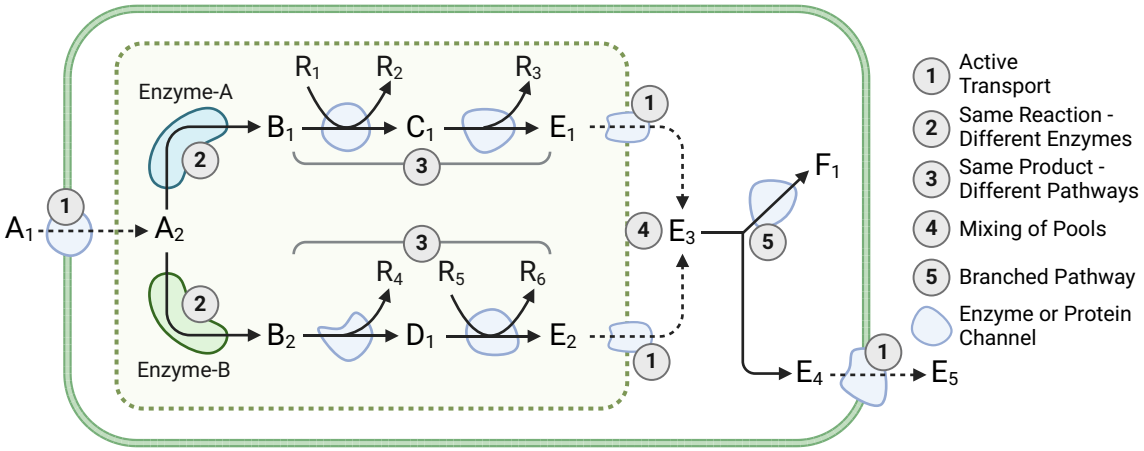

FIGURE 2 A simple, hypothetical biochemical network within a eukaryotic cell, highlighting different processes that lead to isotopic differences in synthesized biomolecules. Focal compounds are denoted by capital letters (A through to F) with numerical subscripts distinguishing between different pools that may differ in isotopic composition. Secondary compounds are denoted as R_n in gray. 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.

Taken together, the $\delta^{13}C$ 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 $\delta^{13}C$ 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 (visualized 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 with the overall average effect of the environment on physiology and therefore carbon acquisition (Larsen et al., 2015; Stahl et al., 2023, Figure 3a,b). From Equation (1), the measured $\delta^{13}C$ 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 characterize the base of the food web contextualized with in situ environmental conditions for different production sources (e.g., Docmac et al., 2017; Søreide et al., 2006). We therefore define measured $\delta^{13}C$-AA values in basal organisms as baseline $\delta^{13}C$-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 centered 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) $\delta^{13}C$ 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 $\delta^{13}C$-AA value minus the mean $\delta^{13}C$-AA value of the basal organism (the non-weighted, within-sample average $\delta^{13}C$-AA value), which we define as the $\delta^{13}C$-AA pattern (Figure 3b). Expressing $\delta^{13}C$-AA patterns via mean-centering is the standard approach first introduced by Larsen et al. (2009, denoted as $\delta^{13}C_N$).

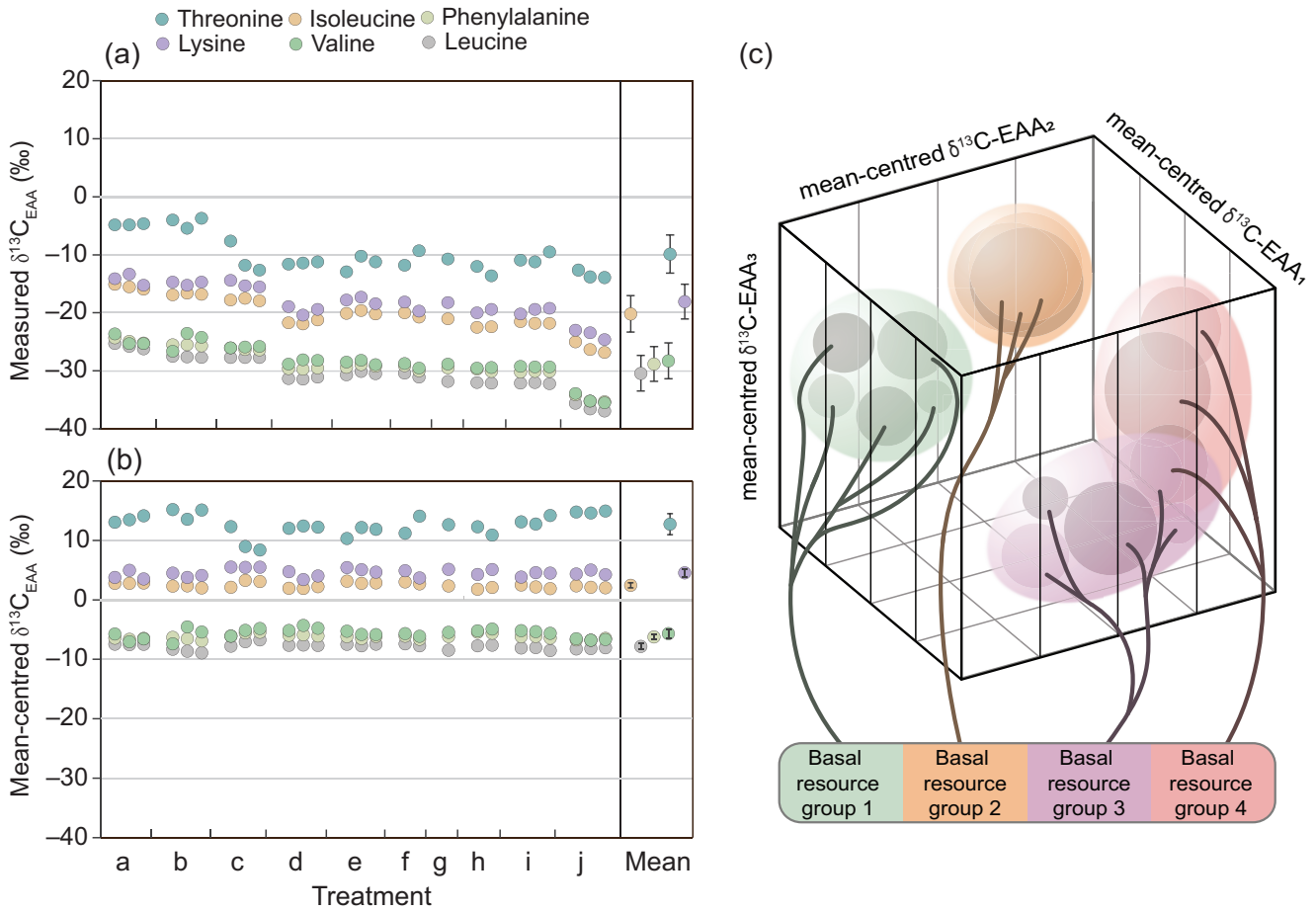

FIGURE 3 The progression from baseline δ^{13}C-EAA values (a) to δ^{13}C-EAA patterns (b) to δ^{13}C-EAA fingerprints (c). (a) The measured δ^{13}C values of six EAAs in the marine diatom *Thalassiosira weissflogii*, cultured under different conditions, show how different environmental conditions influence baseline δ^{13}C-EAA values: (a) 27°C, (b) 18°C, (c) high pH, (d) control, (e) ultraviolet filter, (f) no ultraviolet filter, (g) low irradiance, (h) high irradiance, (i) low pH, (j) low salinity (mean and SD for each EAA across treatments given on the right, data from Larsen et al., 2015). (b) By mean-centering 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 organism groups determines whether the δ^{13}C-EAA patterns constitute δ^{13}C-EAA fingerprints within a study system (illustrated with three EAAs). A basal organism group has a δ^{13}C-EAA fingerprint when that group solely occupies its δ^{13}C-EAA pattern space, for example, Groups 1 and 2. The specificity of the δ^{13}C-EAA fingerprint can be high if subgroups of the basal organisms (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 organism groups exhibit overlap in δ^{13}C-EAA pattern space, for example, Groups 3 and 4.

However, an important constraint is that changes in the combination of AAs result in changes in the absolute offsets in the expressed δ^{13}C-AA pattern, although not the pairwise AA differences.

Isotope fractionations in metabolic networks

While many processes affect measured δ^{13}C-AA values in basal organisms, 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, emphasizing some of the diverse processes that transpire during biosynthesis. δ^{13}C values of synthesized 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 synthesized 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 $\delta^{13}C$ offsets for their respective AAs across taxa. A notable example are the three aromatic AAs that are synthesized 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 synthesize 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 synthesized 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 (Crawford, 1989). Among the aromatic AAs, it is expected that phenylalanine and tyrosine $\delta^{13}C$ 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 utilizing 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 $\delta^{13}C$-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 $\delta^{13}C$-AA patterns among basal organisms. However, ^{13}C kinetic isotope fractionation primarily occurs when the rate limitation of the catalyzing enzyme consists of bond cleaving, formation, or transfers involving carbon atoms. Consequently, not all catalyzed 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 catalyzing 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. Substantial differences in flow rates between pathway steps can lead to deviations from and potentially result in new steady-state conditions. Consequently, the rates of individual biochemical reactions may differ, leading to varying degrees of isotope fractionation at each step (see Hayes, 2001, 2004 for detailed biochemical mechanisms). One key mechanism underpinning flow rates is intracellular compartmentalization (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 synthesize AAs in organelles—involving the active movement of molecules across intracellular membranes which may be associated with additional isotopic fractionation. Clade-specific demands for proteinogenic AAs as precursors for secondary metabolites, energy-yielding substrates, and metabolic donors (Appendix S1: Figures S1–S3) can influence relative flow rates due to branching (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 (Güven et al., 2010) and therefore lack this downstream AA demand. 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 $\delta^{13}C$-AA patterns emerge.

Differences in the synthesis pathways, modulating enzymes, and flow rates between basal organisms that result in distinct $\delta^{13}C$-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 possess only 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., five of the six 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 than 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 synthesize 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.

DISCRIMINATING BASAL ORGANISMS WITH δ^{13}C-EAA FINGERPRINTS

Although all AAs and their δ^{13}C values can be used to distinguish between basal organisms, 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 organisms, the EAA subset of the δ^{13}C-AA patterns, are also preserved (Liu et al., 2018; McMahon et al., 2010; McMahon, Polito, et al., 2015; Wang, Wan, et al., 2019). Published δ^{13}C-EAA patterns have already demonstrated the unique ability to discriminate groups of basal organisms yet offer limited understanding of the underlying processes and potential taxonomic specificity. To develop this fundamental understanding, we build upon our mechanistic framework laid out in section *Factors shaping amino acid δ^{13}C values in basal organisms* by compiling and exploring published δ^{13}C-EAA values of basal organisms at varying levels of phylogeny. We are unable to correct the compiled data for interlaboratory measurement differences due to the lack of common reference materials (see *Minimizing analytical uncertainties in δ^{13}C-AA values*). Nevertheless, we use the data to identify potential underlying mechanisms of basal organism biochemistry, rather than to quantify basal resource use by consumers (see *Applying δ^{13}C-EAA fingerprints in ecological studies* and *Considerations when quantifying basal resource use* for further discussion). We also discuss the potential for some basal organisms to directly assimilate EAAs from the environment. Based on these mechanistic considerations, we refine the definition of δ^{13}C-EAA fingerprints and outline best practices for accurate characterization.

The diagnostic potential of δ^{13}C-EAA patterns among basal organisms

Five EAAs typically reported across ecological and archaeological 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 (See *Isotope fractionations in metabolic networks*, Appendix S1: Figures S1 and S2). 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 δ^{13}C offsets. Bacteria generally exhibit significant metabolic redundancy and flexibility in synthesizing EAAs (Cotton et al., 2020), but it is difficult to predict how this influences δ^{13}C-EAA patterns. Based on biosynthesis pathways, phenylalanine emerges as the most likely candidate to cause consistent divergence in δ^{13}C-EAA patterns between plants and algae, and bacteria and fungi. In terms of differential flow rates influencing δ^{13}C-EAA patterns, there are two systematic differences between broad basal organism groups. The first is intracellular compartmentalization within eukaryotes that affects the synthesis pathways of the five EAAs (Figure 1), potentially separating bacterial δ^{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 δ^{13}C-EAA patterns. As lignin is relatively depleted in ^{13}C compared

with 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 (Domozych, 2019; Kottom et al., 2017; 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 utilization 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 analyzed data of 680 samples from 20 ecological and archaeological studies (Figure 4, Appendix S2). We applied linear discriminant analysis (LDA), which aims to separate different groups of basal organisms based on their $\delta^{13}C$-EAA patterns. LDA does this by maximizing the differences between groups while minimizing within-group variability, providing EAA-specific weightings for group separation. To quantify the extent of overlap between groups, we calculated pairwise Bhattacharya coefficients (BCs, Bhattacharyya, 1946) on the LDA-transformed data (see Appendix S2 for details). 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 BC 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 $\delta^{13}C$-EAA patterns express considerable variation, substructuring is observed among the three clades of macroalgae. Brown (Phaeophyta) and red macroalgae (Rhodophyta) $\delta^{13}C$-EAA patterns appear to separate (median overlap 0.35), but green macroalgae (Chlorophyta) occupy the overlapping $\delta^{13}C$-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 $\delta^{13}C$ offsets. This is similarly the case when contrasting seagrasses with microalgae (Appendix S2: Figure S1b), where the $\delta^{13}C$-EAA patterns of phytoplankton diverge between freshwater and marine biomes (median overlap of 0.33). Terrestrial plant $\delta^{13}C$-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 $\delta^{13}C$-EAA values (See *Factors shaping amino acid $\delta^{13}C$ values in basal organisms*). Some individual C_3 plants express similar $\delta^{13}C$-EAA patterns to CAM plants (Figure 4c). We explored substructuring of $\delta^{13}C$-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 $\delta^{13}C$-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 $\delta^{13}C$ offsets (Figure 4d). Despite phenylalanine separating plants from other basal organism groups, phenylalanine along with valine contribute little to $\delta^{13}C$-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 conclude that $\delta^{13}C$-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 are separated by phenylalanine $\delta^{13}C$ 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 S1b). Phenylalanine contributed little to $\delta^{13}C$-EAA pattern variability within vascular plant phylogeny, which is consistent with the ubiquity of lignin synthesis in this group. The observation that $\delta^{13}C$-EAA pattern distinctions can be observed not only with phylogeny, but also by ecosystems such as marine versus freshwater algae (Appendix S2: Figure S1a,b) and similarities between arid climate-adapted plants (Figure 4d), further highlights how consistent phenotypic expressions may contribute to $\delta^{13}C$-EAA pattern variation. Several other studies have observed $\delta^{13}C$-EAA pattern distinctions between organs of individual plants, that is, roots, seeds, and leaves (Jarman et al., 2017; Larsen, Ventura, et al., 2016; Lynch et al., 2011), demonstrating that structural differences can underpin $\delta^{13}C$-EAA patterns within the same individual.

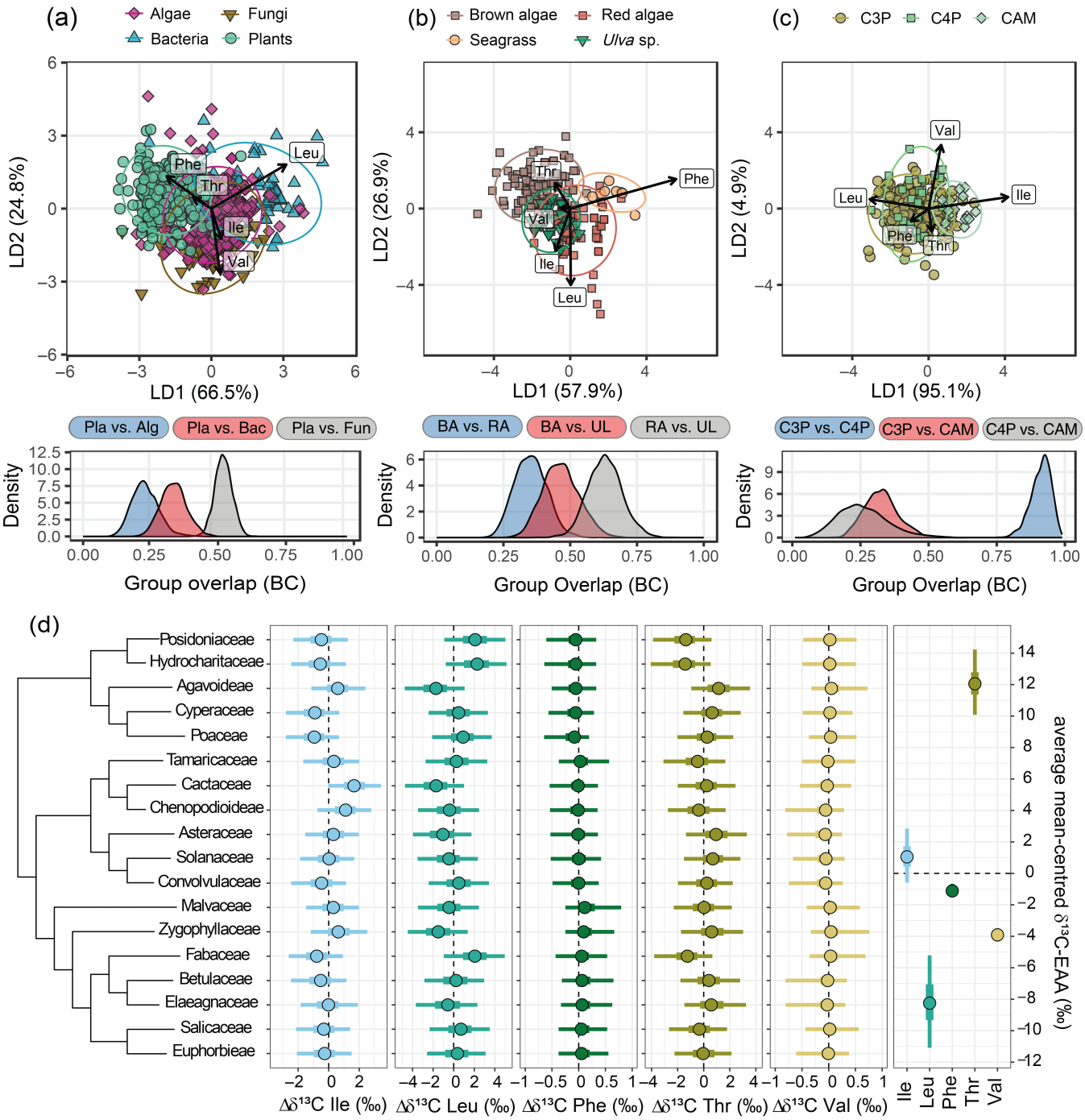

FIGURE 4 Linear discriminant (LD) analysis of mean-centered δ^{13}C-EAA values in basal organisms compiled from the literature (see Appendix S2). Upper subplot panel: LD scores for individual samples, with distinct symbols denoting each group. Lower subplot panel: posterior distributions of group pair 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 basal organism taxa: (a) heterotrophic bacteria (Bac), plants (Pla), algae (Alg), and fungi (Fun); (b) brown macroalgae (BA), red macroalgae (RA), green macroalgae (UL, represented by *Ulva* sp.), and seagrasses; (c) C_3 plants (C3P), C_4 plants (C4P), and CAM (crassulacean acid metabolism) plants, containing the two cacti species *Cylindropuntia* sp. and *Opuntia* sp. For visual clarity, LD weighting coefficients for each EAA were multiplied by 8. The BCs for seagrass are not shown to avoid overcrowding; see Appendix S2 for their posterior estimates. (d) Modeled mean-centered 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-centered δ^{13}C CIs for phenylalanine and valine fall within the median circles.

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—that is, 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 & Skarlato, 2013; Selosse & 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, Liew, & Hopkins, 2022), and therefore requires knowledge of the occurrence, degree, and flexibility of facultative prototrophy in different basal resource groups.

Assimilating AAs opportunistically from the external environment is energetically efficient compared with synthesizing them de novo (Morrissey et al., 2023); however, basal organisms must have the necessary membrane proteins that are energetically expensive to synthesize and maintain. The capacity for AA assimilation among 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 (D'Souza et al., 2014; Heizer et al., 2006), suggesting that demand for external AAs will be substantial and highly competitive. Functional specialization 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 (D'Souza et al., 2014; Heizer et al., 2006). 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 specialized 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; Firmin et al., 2022; Selosse et al., 2016), beyond the limited cases of carnivory and hemi-parasitism (Giesemann & Gebauer, 2022; Selosse & Roy, 2009). AA membrane transport proteins have been characterized in several species of plant roots, the prerequisite for direct uptake of external AAs (Moe, 2013; Näsholm et al., 2009). Although mixotrophic protists that phagocytose prey have long been recognized (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 & 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 & 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, Liew, & Hopkins, 2022). Labelling experiments demonstrate however that uptake of external AAs occurs during exponential but not stationary growth phases in fungi (Martin-Perez & Villén, 2015). For bacteria, specialized adaptations suggest that external AAs will benefit only those species that readily utilize labile dissolved organic carbon (Dang et al., 2022; Morrissey et al., 2023). Average dissolved AAs in soils and aquatic environments typically occur in low concentrations of 0.01–50 μM and 1–10 μM, respectively (Kielland, 1994; Lytle & Perdue, 1981, cf. 130–840 μM in "low" AA concentration treatment in Arsenault, Liew, & Hopkins, 2022). 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.

Although 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 labeled EAA media under natural growth conditions could provide insights into the metabolic dependencies of facultative EAA-prototrophs on external EAAs.

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 of 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., Arthur et al., 2014; Larsen et al., 2012; 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 baseline rather than mean-centered 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 & Newsome, 2019). Considering 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 multivariate δ^{13}C-EAA pattern space** that is **solely occupied** by a group or collection of similar **basal organisms**, encompassing 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 the multivariate space of δ^{13}C-EAA patterns (as defined in Table 1, Figure 3). By limiting it to the minimum space occupied, arbitrary overlaps between basal organisms are excluded. However, as sole occupancy of δ^{13}C-EAA pattern space is comparative, it depends upon the presence or absence of other basal organisms in an ecosystem (shown in Figure 3c, basal organism Groups 3 and 4) or its relevance to the consumer (See *Applying δ^{13}C-EAA fingerprints in ecological studies*). A priori understanding of a consumer's ecology and its ecosystem underpins which basal organism 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 organism 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 (See *The diagnostic potential of δ^{13}C-EAA patterns among basal organisms*, Figures 3a,b and 4d, Appendix S3: Figure S1). 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 organism groups are characterized (Arsenault, Thorp, et al., 2022; Arthur et al., 2014; Ayayee et al., 2015; Macartney et al., 2020; McMahon, McCarthy, et al., 2015; Rowe et al., 2019; Stubbs et al., 2022; Wall et al., 2021). 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 (See *Factors shaping amino acid δ^{13}C values in basal organisms*) 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.

Optimal characterization of δ^{13}C-EAA fingerprints

Defining δ^{13}C-EAA fingerprints requires a conscientious approach to basal organism sampling and analysis. The

characterization of δ^{13}C-EAA fingerprints involves accurate representation of a particular basal organism group in an ecosystem, its natural variation, and its relevance to the studied consumer. For optimally characterizing δ^{13}C-EAA fingerprints, the following considerations are important:

1. Basal organism samples should accurately represent the taxonomic group under investigation in the studied ecosystem. This precludes complex composites such as particulate organic matter (POM) filtrates, microalgal and bacterial mats, or partially degraded materials (detritus) that are contaminated with feces, degraded organic matter, bacteria, etc. Further, composites average over a diversity of species, preventing specific characterization. In some cases, more specific δ^{13}C-EAA fingerprints for individual species can be obtained by extraction from composite samples and cultivation under controlled laboratory conditions. Systematic characterization of δ^{13}C-EAA fingerprints in singularly cultured basal organisms would establish the extent to which basal organisms 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 analyzed for verification, as some basal organisms might display different δ^{13}C-EAA patterns in situ compared with cultures (Vane et al., 2023).
2. Tissue samples of specialist primary consumers (e.g., zooplankton or herbivorous fishes) are often used as a surrogate for specific δ^{13}C-EAA fingerprints of basal organisms (e.g., McMahon et al., 2016; Skinner et al., 2021; Vane et al., 2018). However, complete dependency of a primary consumer on basal resources of one specific clade of basal organisms is unlikely due to incidental EAA assimilation from other sources (e.g., functionally similar basal organisms, detritus, associated bacteria and meiofauna in macroalgal turfs, Nicholson & Clements, 2023).
3. The extent to which unique δ^{13}C-EAA fingerprints can be characterized depends on the number of EAAs measured in basal organisms and metazoan tissues due to analytical limitations (See *Temporal resolutions within consumer tissues* and *Minimizing analytical uncertainties in δ^{13}C-AA values*). In most proteinaceous soft tissues six to seven EAAs can be measured, but this number is reduced in mineralized tissues such as biogenic calcites due to lower EAA concentrations (McMahon et al., 2018; Vokhshoori et al., 2022). It is advisable to reliably measure, and report, as many EAAs as possible (See *Minimizing analytical uncertainties in δ^{13}C-AA value*) to increase the discriminatory power of δ^{13}C-EAA patterns.

Directly visualizing whether δ^{13}C-EAA patterns of select basal organism groups solely occupy their isotopic space, and therefore are a δ^{13}C-EAA fingerprint (See *From δ^{13}C-EAA patterns to fingerprints*, Figure 3c), is not feasible due to the high dimensionality of the data. Visualizing multiple pairwise biplots of mean-centered δ^{13}C-EAA values results in significant information loss and can be difficult to interpret. Dimension reduction approaches used to visualize δ^{13}C-EAA patterns include principal component analysis (PCA, which maximizes total variation across the dataset) and LDAs (See *The diagnostic potential of δ^{13}C-EAA patterns among basal organisms*). While LDAs may seem more effective to identify distinctions between δ^{13}C-EAA patterns as it aims to maximize group separation, PCA can outperform LDA in separating groups when sample sizes are small (Martinez & Kak, 2001; for a comparison of the two approaches see Appendix S4). The distinctions between δ^{13}C-EAA patterns can be objectively quantified using, for example, BCs (Bhattacharyya, 1946, see *The diagnostic potential of δ^{13}C-EAA patterns among basal organisms*, Figure 4a–c, Appendix S2). Quantifying δ^{13}C-EAA pattern distinctions not only improves statistical clarity for defining δ^{13}C-EAA fingerprints but also facilitates more direct comparisons between studies that measure different suites of EAAs.

TRACING BASAL RESOURCES FROM A CONSUMER PERSPECTIVE

Organisms consume the basal resources synthesized 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 organisms 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 synthesized the basal resources using δ^{13}C-EAA fingerprints. Prior knowledge of the consumer's dietary niche is essential to characterize relevant basal organisms, and to determine the extent of the distinction and specificity with which they 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.

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

δ^{13}C-EAA fingerprints can differentiate basal organisms across broad taxonomic groups and finer clades (See *Discriminating basal organisms with δ^{13}C-EAA fingerprints*). 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 organisms 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 basal resources originating from different groups of basal organisms, or distinguishing among closely related clades such as phytoplankton groups (McMahon, McCarthy, et al., 2015; Stahl et al., 2023; Vane et al., 2023), although such fine-scale distinctions may not always be informative depending on the ecological setting. For example, the fine resolution of distinguishing between various clades within phytoplankton is diminished when contrasted against other major basal organism groups such as bacteria, fungi and macroalgae (Vane et al., 2023). After thoroughly characterizing δ^{13}C-EAA fingerprints within the research framework, their variation must 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 organism or incomplete characterization due to limited replication or sampling. Biases during isotopic analysis can also lead to measurement offsets between consumer tissues and basal resources (See *Minimizing analytical uncertainties in δ^{13}C-AA value*). These considerations are important for reliable quantifications of proportional basal resource use by the consumer (See *From qualifying to quantifying basal resource use*).

Many researchers rely on existing training datasets, that is, basal organism δ^{13}C-EAA values characterized 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 δ^{13}C-EAA patterns are highly conservative with broad ecosystem applicability, a questionable assumption at broad taxonomic scales. As elaborated in section *Discriminating basal organisms with δ^{13}C-EAA fingerprints*, 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 patterns vary among seeds, roots, and leaves (Jarman et al., 2017; Larsen, Ventura, et al., 2016; Lynch et al., 2011), 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 resource origins 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 (See *Minimizing analytical uncertainties in δ^{13}C-AA value*). While researchers often supplement measured δ^{13}C-EAA values of basal organisms in the studied ecosystem with external training data (e.g., Arsenault, Thorp, et al., 2022; Arthur et al., 2014; Ayayee et al., 2015; Macartney et al., 2020; Rowe et al., 2019; Stubbs et al., 2022; Wall et al., 2021), for accurate inferences it is advisable to characterize study-specific δ^{13}C-EAA fingerprints of relevant basal organisms measured in situ. Going forward, a δ^{13}C-EAA fingerprint library could streamline this process, if built on widely accepted international reference materials and standardized methodologies (See *Minimizing analytical uncertainties in δ^{13}C-AA value*). Such a library would be invaluable in addressing large-scale ecological questions over various spatiotemporal scales.

Consumers with (endo)symbionts

The direct assimilation of basal resources by consumers can be compounded by the occurrence of symbiotic relationships. Endosymbionts can supplement hosts with EAAs synthesized de novo, particularly when the host specializes on nitrogen or nutrient-poor diets. Examples include aphids and other plant sap feeding insects with sugar dominated diets (Akman Gündüz & Douglas, 2009), or detrital consumers, such as earthworms, springtails, and termites (Ayayee et al., 2015; Larsen, Pollierer, et al., 2016). EAA supplementation can vary dynamically depending on dietary availability and digestibility, leading to trade-offs. For instance, experimentally increasing indigestible fiber content in enchytraeids' diets increased EAA supplementation by gut symbionts, but reduced enchytraeid growth (Larsen, Pollierer, et al., 2016). However, digestive anatomy and physiology is important when considering gut microbiome EAA supplementation. For example, in most vertebrates, AA assimilation occurs primarily in the small

intestine, and therefore, the distinction between small and large intestinal microbes is critical when characterizing microbial δ^{13}C-EAA patterns for EAA absorption (MacRae et al., 1997; van der Wielen et al., 2017). As such, fecal or cecal extracted microbiota poorly represent the microbial communities involved in EAA provisioning in the small intestine. Coprophagy can add further complexities as this can enhance "apparent" gut microbial contributions as demonstrated through controlled feeding studies (see Torrallardona et al., 2003).

In marine environments, mixotrophic holobionts such as corals, mollusks, and sponges rely on a complex community of symbionts in addition to heterotrophic feeding (Pita et al., 2018; Skinner et al., 2022). 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 (Fine & Loya, 2002; Schlichter et al., 1995). 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 characterization 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 POM, a proxy for phytoplankton (Fox et al., 2019; Wall et al., 2021) and other free-living dinoflagellates (Stahl et al., 2023). However, similar characterizations 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 characterized, despite the possibility to cultivate gut microbes from model organisms such as *Drosophila* (Erkosar et al., 2013, but see Besser et al., 2023 for extracting microbial biomass from fecal materials for δ^{13}C-AA analyses). Currently, researchers predominantly rely on training data from disparate terrestrial bacteria to identify gut microbial EAA supplementation (e.g., Arthur et al., 2014; Ayayee et al., 2015; Stubbs et al., 2022), which may yield inaccurate quantifications (see *Applying δ^{13}C-EAA fingerprints in ecological studies*). 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, Ventura, et al., 2016; 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 characterization 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 characterized, they could aid in identifying and quantifying changes in EAA symbiont provisioning to their host.

BEYOND δ^{13}C-EAA FINGERPRINTS

Consumers may rely on a variety of basal resources whose origins cannot be distinguished using δ^{13}C-EAA fingerprints. For example, researchers might aim to differentiate contributions from phylogenetically similar understory versus canopy vegetation, or sea-ice microalgae versus pelagic phytoplankton (de la Vega et al., 2019; Tejada et al., 2020). Spatial or environmental segregation of these basal organisms within the ecosystem (e.g., ice-algae vs. phytoplankton), or differences in carbon fixation machinery (e.g., C_3 vs. C_4 plants) can result in disparate baseline δ^{13}C-EAA values between basal organism groups, despite their δ^{13}C-EAA patterns being similar. Even when basal organisms lack distinct δ^{13}C-EAA patterns, they may still have different baseline δ^{13}C-EAA values due to spatial, environmental, or physiological factors (See *Conceptualizing amino acid δ^{13}C values in basal organisms*, Figure 1). In such cases, using measured δ^{13}C-EAA data from consumer tissues, rather than mean-centered δ^{13}C-EAA values, can help differentiate the contributions of these basal resources to food webs, assuming all resources can be adequately sampled in situ. By applying multivariate analyses to baseline δ^{13}C-EAA values, researchers have distinguished between freshwater algae, marine algae, terrestrial matter, and detrital material simultaneously in consumers (Johnson et al., 2019; McMahon et al., 2016; Vane et al., 2018, 2023). Incorporating δ^{13}C-NEAA values from consumer tissues 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, Wang, & Wan, 2022) 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 conceptualizations from section *Factors shaping amino acid δ^{13}C values in basal organisms*, 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.

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 synthesized de novo by the organism and those assimilated from the dietary tract (ca. Figures 1–5). 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 S3 for a detailed metabolic network). For instance, lipids and short-chain fatty acids are generally depleted in ^{13}C compared with proteins and carbohydrates (Deniro & Epstein, 1977; Melzer & 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, Wang, & Wan, 2022). Additionally, dietary sourced AAs may experience fractionation during their catabolic processing within the microbiome of the abdominal cavity. The consumer 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 S3). 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 destined for catabolic processing are low, ranging from 20% to 50% except for 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 center of AA degradation and synthesis—they serve various functions, including as building blocks for proteins and precursors for non-proteinogenic metabolites (Burrin & Stoll, 2009, Figure 5). Excess dietary NEAAs are converted into energy dense molecules such as fats and glycogen, which can later be catabolized into glucose as needed. Although the precise ratio of dietary to synthesized 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, 2017).

Exploring full δ^{13}C amino acid datasets

Many factors encompassing diet, digestive physiology, metabolism, and life history traits influence δ^{13}C-NEAA values in consumers (Larsen, Fernandes, et al., 2022). Unraveling these complex interactions necessitates a comprehensive approach, especially when trying to distinguish metabolic from dietary effects. To isolate the factors contributing to δ^{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 characteristics among consumers like digestive physiology, metabolism, and life histories (Larsen, Wang, & Wan, 2022). Intriguingly, the most consistent and robust insights into δ^{13}C-NEAA data have emerged from human studies when interpreted in concert with measured δ^{13}C-EAA values and δ^{13}C-EAA patterns in human tissues (Choy et al., 2013; Corr et al., 2005; Johnson et al., 2021; Yun et al., 2018, 2020). Epidemiological studies have shown that δ^{13}C-NEAA values in humans can vary with specific food compositions, but have so far explored only a limited spectrum of human diets (Choy et al., 2013; Johnson et al., 2021; Yun et al., 2018, 2020). This suggests that variations in δ^{13}C-NEAA values can deepen our understanding of the complex interplay between consumer biology, and their diverse dietary sources.

To broaden our perspective on integrating δ^{13}C-NEAA values for understanding basal resource use, we assembled archaeological δ^{13}C-AA data from human bone collagen and hair keratin samples, covering a period of 6500 years from diverse geographical locations (eight studies, $n = 52$; see Appendix S4 for details). This dataset includes δ^{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 were derived from four major protein sources: freshwater, marine,

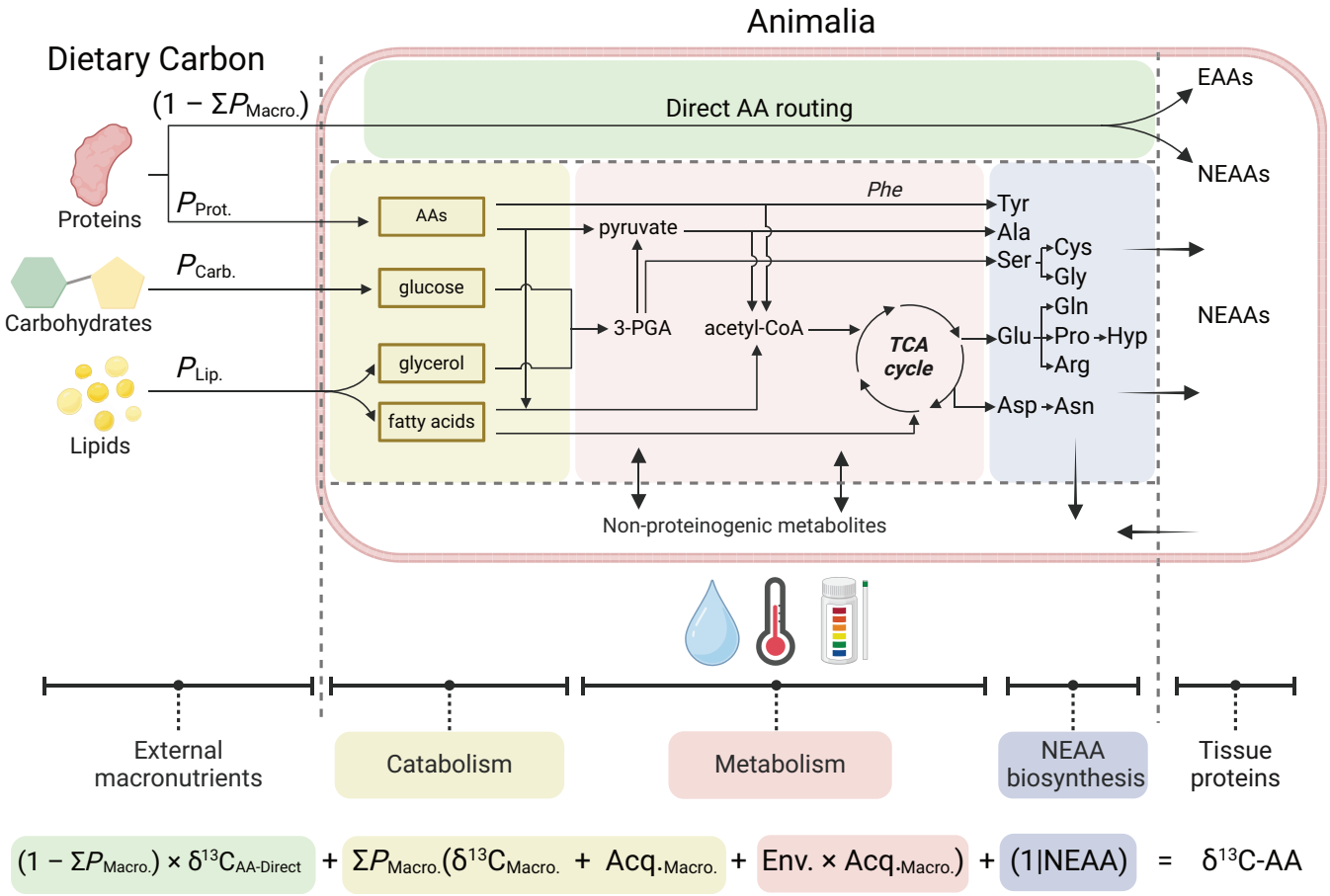

FIGURE 5 Conceptual schematic incorporating macronutrients (Macro.) such as proteins; Prot., carbohydrates; Carb., and lipids; Lip.), metabolic processes, and environmental effects that contribute to the δ^{13}C-AA values in animals (ca. Figure 1). Metabolic processes are divided into macronutrient catabolism, central metabolism including glycolysis and the tricarboxylic acid (TCA) cycle, and the biosynthesis of the non-essential AAs (NEAAs, 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) that can be utilized for proteinogenic or non-proteinogenic purposes. All the essential AAs (EAAs) are assumed to be routed directly from dietary proteins ($\Sigma P_{\text{Macro.}} = 0$, where P is proportional contribution). A fraction of the dietary NEAAs may be routed directly to tissue proteins ($1 - \Sigma P_{\text{Macro.}}$), 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, $\delta^{13}C_{\text{Macro.}}$, and fractionation during carbon acquisition, $\text{Acq.}_{\text{Macro.}}$, as they are converted to NEAA-precursors. As the catabolic networks are different for the three macronutrients (Appendix S1: Figure S3), the effect of environment, Env., will likely induce different physiological responses in isotopic fractionations ($\text{Env.} \times \text{Acq.}_{\text{Macro.}}$). The contributions of different macronutrients to NEAA synthesis ($\Sigma P_{\text{Macro.}} = P_{\text{Prot.}} + P_{\text{Carb.}} + P_{\text{Lip.}}$) 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 catabolized and re-enter the central metabolism. The metabolic pathways are summarized based on Stryer et al. (2019, see Appendix S1: Figure S3 for a detailed metabolic network). 3-PGA, 3-phosphoglyceric acid. The illustration was created with BioRender.com.

terrestrial C_3 plants, and terrestrial C_4 plants. 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-centered δ^{13}C-AA data (Figure 6a,b), with results showing relatively strong separation among the four protein sources. Most AAs align with PC1, which differentiates populations along a terrestrial–aquatic (freshwater and marine) axis, while PC2 distinguishes between C_3 and C_4 protein sources and is primarily driven by the δ^{13}C contrast between proline and aspartate. These distinctions are further pronounced by combining measured δ^{13}C-AA values—data that comprises the individual δ^{13}C-AA offsets combined with the δ^{13}C bulk baselines—

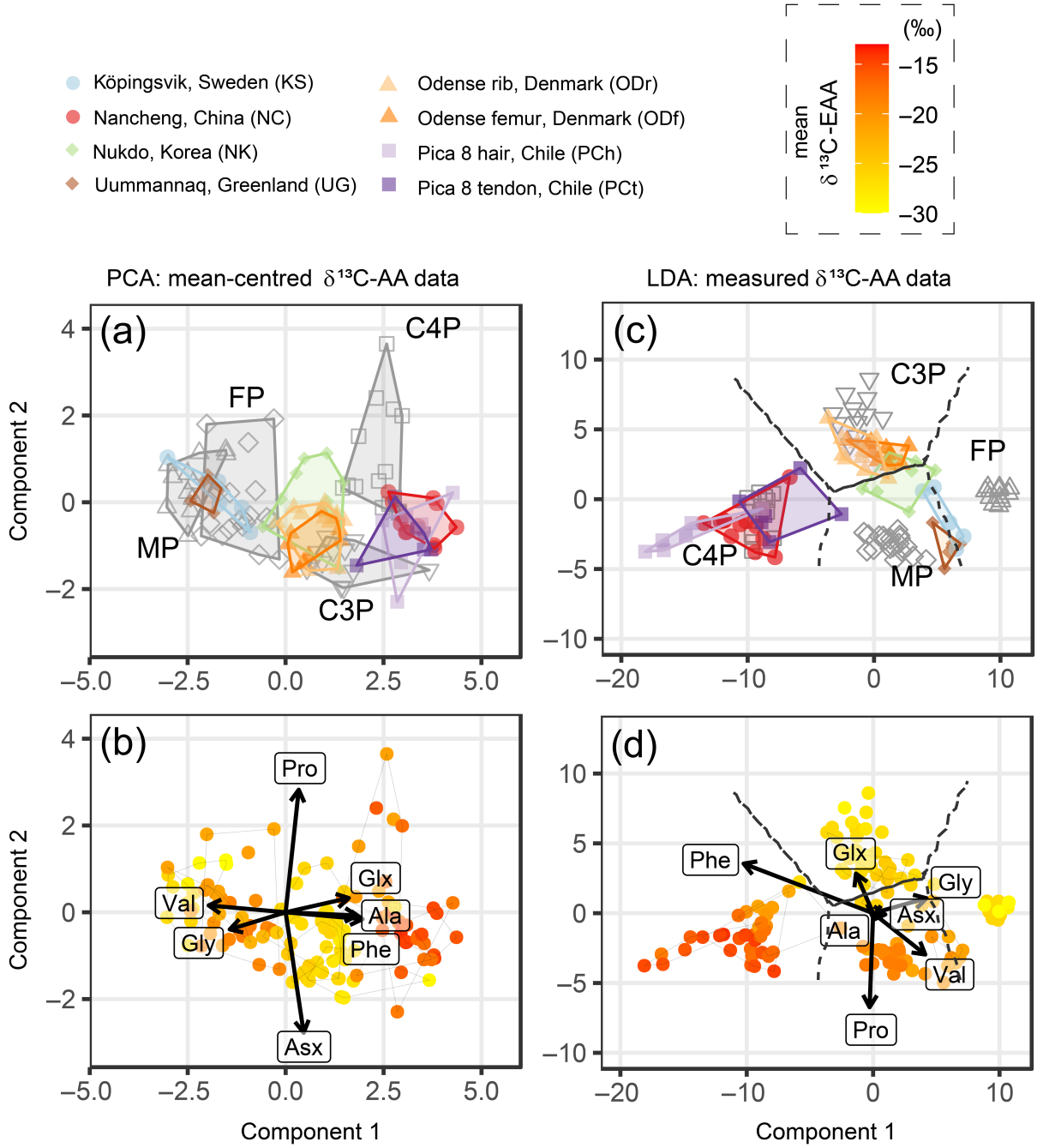

FIGURE 6 Ordination analyses using $\delta^{13}C$ values of alanine (Ala), aspartate/asparagine (Asx), glycine (Gly), glutamate/glutamine (Glx), phenylalanine (Phe), proline (Pro), and valine (Val) extracted from archaeological human collagen and keratin samples. Sites include: Köpingsvik, Sweden (KS, Mesolithic and Middle Neolithic), Nancheng, China (NC, Proto-Shang), Nukdo, Korea (NK, Late Bronze Age), Uummannaq, Greenland (UG, sixteenth–seventeenth centuries), Odense, Denmark (ODr/ODf, Medieval), and Pica 8, Chile (PCh/PCt, Late Intermediate Period). Subplots (a) and (b) display the first two principal components (PC1 and PC2 explain 54.7% and 19.6% of the variance, respectively), based on $\delta^{13}C$-AA values centered to the within-sample mean EAA values ($\delta^{13}C\text{-}AA_{mean}$ of Phe and Val). Subplots (c) and (d) show the first two linear discriminants (LD1 and LD2 explain 76.6% and 21.8% of the variance, respectively) based on measured $\delta^{13}C$-AA data. Subplots (a) and (c) categorize individuals according to their respective populations, while the color-gradient subplots (b) and (d) 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 gray symbols and originate from Belize, Brazil, Bulgaria, Greenland, Guatemala, Japan, Serbia, and Romania (data from Colonese et al., 2014; Honch et al., 2012). Populations with less certain diets are plotted with distinctly colored symbols and polygons. 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.

with LDA that maximizes group separation while minimizing intra-group variation. The LDA highlights significant variability in the contribution of different AAs to group separation within measured δ^{13}C-AA values. Phenylalanine and valine again distinguish between terrestrial and aquatic sources (Honch et al., 2012; Larsen et al., 2013), while proline and glutamate separates C_3 diets (Figure 6c,d). Glycine plays a key role in differentiating freshwater protein 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 δ^{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 aspartate and source effects in case of proline (Figure 6b,d), can separate C_3 and C_4 plant sources when δ^{13}C-EAA patterns cannot (Figure 4c). To delve deeper into the multifaceted factors that drive variability in δ^{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 δ^{13}C-NEAA values in consumers.

When using multivariate analyses to differentiate dietary sources, the choice between measured and mean-centered δ^{13}C-AA values depends on the specific context of the study. Measured δ^{13}C-AA values in consumer tissues are particularly effective when the protein sources originate from distinct biomes, such as terrestrial versus aquatic, or when there are significant differences in baseline δ^{13}C-AA values between the dietary sources, for example, C_3 versus C_4 vegetation or freshwater versus marine. In these cases, incorporating measured δ^{13}C-AA values alongside δ^{13}C-AA patterns in multivariate analyses can compensate for the limitations of each approach while leveraging their strengths. This synergy is most effective when the variability in isotopic baselines between biomes (intergroup variability) is substantially greater than the variation within a single biome (intragroup variability). A case in point is the meta-analysis involving archaeological human samples presented above, and ecological studies that characterize or approximate baseline δ^{13}C-EAA values directly from basal organisms in situ such as Vane et al. (2018, 2023) and Johnson et al. (2019).

However, it is important to recognize the limitations of using measured δ^{13}C-AA values in multivariate analyses. Baseline δ^{13}C-AA values are sensitive to environmental fluctuations, making them context-dependent (McMahon et al., 2016; Vane et al., 2023). When baseline δ^{13}C-AA values show only subtle distinctions between basal organisms, comprehensive sampling strategies become crucial. Seasonal or spatial sampling can help constrain the variation in baseline δ^{13}C-AA values for each basal organism group, providing a more stable context for analysis (See *Interpreting mixing model output*, Vane et al., 2023). This allows for a more reliable estimation of baseline δ^{13}C-AA values in environments where protein sources within a biome are not sharply delineated. Researchers may face additional challenges in situations where comprehensive sampling is not feasible, such as when studying historical/archaeological samples, modern environments with temporally dynamic resources (e.g., lakes, coastal environments), mobile consumers (e.g., migrating birds, insects) that assimilate AAs over extensive spatial areas, or when confronted with logistical constraints (e.g., costs). These sources often present gaps in spatiotemporal data, limited sample sizes, material degradation, or incomplete records, which can complicate the construction of a robust analytical framework. In these cases, auxiliary data like climatic records, historical/archaeological contexts, or alternative sampling strategies may provide complementary information for constraining protein sources and inform their analyses when isotope data alone are insufficient.

CONSIDERATIONS FOR USING ARCHIVAL TISSUES

AAs are highly persistent in preserved and metabolically inactive tissues. This persistence together with their ability to track biosynthetic origins makes AAs a powerful tool to investigate changes in basal resource use within and between consumer populations, and over time scales ranging from seasons to millennia. Tracing how a consumer's use of basal resources varies across different habitats and time periods depends on the rate at which AAs are assimilated and replaced in various tissues (i.e., tissue turnover rate), and the preservation of those tissues (Boecklen et al., 2011). Stable isotope analysis of AAs holds a distinct advantage over that of composite bulk samples because δ^{13}C-AA values are less affected by preservation conditions, such as the incorporation of exogenous carbon into the bulk tissue via chemical

preservatives or diagenic contaminants. Nonetheless, the ability to use AAs to reconstruct past basal resource use of individual consumers relies on the preservation and integrity of tissue samples. In this section, we discuss tissue characteristics that enable specific basal resource use reconstructions over time and space.

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. While direct measurements of AA turnover rates in tissues are scarce, studies have shown that they often closely match turnover rates measured with bulk $\delta^{13}C$ values. This similarity suggests that existing knowledge of bulk $\delta^{13}C$ turnover rates can be used to estimate the temporal resolution of $\delta^{13}C$-AA-based reconstructions of basal resource use (see Martínez del Rio et al., 2009 for a comprehensive review). AAs in blood plasma and soft tissues, such as liver and muscle, can be turned over within days to months depending on tissue metabolism, age, size, or species (Boecklen et al., 2011; Hesse et al., 2022; Robinson et al., 2011; Vander Zanden et al., 2015). Many hard and semi-hard tissues such as bones and ligaments are also remodeled throughout life at different rates varying with age, gender, physiology, and pathological conditions (Hadjidakis & Androulakis, 2006). By analyzing different skeletal tissues with contrasting turnover rates, such as bone collagen and hair keratin, basal resource use can be reconstructed over different time periods, ranging from weeks to decades (Fahy et al., 2017; Matsubayashi & Tayasu, 2019; Tieszen, 1983). Inert keratin excrescences such as hair, nails, and feathers capture longitudinal basal resource use over seasons as they grow continuously until shedding. 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 increments of metabolically inert calcium carbonate structures such as bivalve shells, coral skeletons, and fish otoliths (Borelli et al., 2001; Edeyer et al., 2000; Falini et al., 2015; Payan et al., 1999), and similarly so with chitinous cephalopod beaks and cartilaginous vertebrae of sharks (Cherel et al., 2009, 2019; Magozzi et al., 2021). However, material wear due to mechanical abrasion can limit the temporal window of information contained in structures such as beaks and baleen (Aguilar & Borrell, 2021).

The temporal resolution of incrementally grown tissues 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 typically low, generally ranging between 0.5% and 2%, requiring large 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, reducing 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.

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 (Collins et al., 2002; Grupe, 1995; Iglesias-Groth et al., 2011). Several degradation indicators such as AA stereoisomer ratios, and stable $\delta^{13}C$-AA and $\delta^{15}N$-AA values, suggest that high density carbonate matrices such as egg and bivalve shells remain inert for at least 10,000 years under favorable conditions (Engel et al., 1994; Johnson et al., 1998; O'Donnell et al., 2007; Silfer et al., 1994; Tuross et al., 1988). 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 tissue surfaces and should be removed prior to analysis (e.g., mechanically, by dilute acid washing, or sonication, Engel et al., 1994). This can be challenging in porous structures such as coral skeletons and damaged 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 typically persist on geological timescales 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 (Brock et al., 2012).

Soft tissues that readily degrade are best stored 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 $\delta^{13}C$-AA or $\delta^{15}N$-AA values (Chua et al., 2020;

Durante et al., 2020; Strzepek et al., 2014), but alterations have been observed for samples stored for prolonged periods of up to 27 years (Durante et al., 2020; Hannides et al., 2009; Hetherington et al., 2019; 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). Preservation chemicals likely affect tissue integrity by impacting the peptide and protein bonds, leading to unstructured AA leaching and affecting δ^{13}C-AA values of the tissue due to mass-based diffusion differences long term. 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.

MINIMIZING ANALYTICAL UNCERTAINTIES IN δ^{13}C-AA VALUES

Carbon isotope analysis of AAs poses greater methodological challenges and analytical error potential than bulk stable isotope analysis. Bulk isotope analysis typically consists of weighing dried tissue that is then combusted in the elemental analyzer, although some samples may require pretreatment. The procedures for AA analysis are more complex as AAs must first be extracted and isolated from the diverse compounds within a sample (see Figure 7). Isotopic measurement can be done using a gas or liquid chromatograph interfaced to a combustion reactor and isotope ratio mass spectrometer (GC-IRMS or LC-IRMS). For GC-IRMS, polar charged AAs need to be chemically modified for evaporation (Silverman et al., 2022). Conversely, AAs can be analyzed directly with LC-IRMS but analytical sensitivity is comparatively low (Dunn et al., 2011; Smith et al., 2009). Monitoring the consistency and stability of compound-specific isotope measurements requires the use of reference materials. With 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.

Analytical workflow

To assess the stable isotope composition of individual AAs, samples are first dried and homogenized, then subjected to 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). Acid hydrolysis also disrupts additional chemical bonds, particularly those within the EAA tryptophan, yielding a complex mixture of AAs alongside 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. Purification methods vary, depending on the type of tissue analyzed and chemical protocol employed. Carbonate AAs in bone collagen must be removed due to their susceptible to diagenetic processes and different turnover rates than collagen (Lambert & Grupe, 1993; Stafford et al., 1988). The decalcification process involves soaking whole bones in a mild acid (Figure 7: Step 1, Brault et al., 2014; Sealy et al., 2014), while other biogenic carbonates undergo homogenization, acid hydrolysis, and purification using cation exchange resins (Figure 7: Step 5). For samples rich in secondary metabolites, such as soils, plants, and algae, purification with cation exchange resins may also be necessary. Large particulates in hydrolyzed samples are removed through glass wool filtration, while chemical extraction removes lipophilic compounds (Figure 7: Step 4). The samples undergo drying after purification, followed by the addition of molecularly similar internal reference compounds to account for potential AA losses or isotope effects (Figure 7: Step 6). Prior to GC-IRMS analysis, AAs undergo chemical modification—known as derivatization—increasing their volatility and enabling chromatographic separation. This process is done chemically by specifically targeting AA functional groups (Figure 7: Step 7). Following derivatization, internal reference compounds with known isotope values are added to the sample. After GC-IRMS analysis, chromatograms must undergo quality control and assurance to evaluate consistency of AA peak integration and co-elution issues (Figure 7: Step 9). Co-elution can create substantial measurement errors when an AA peak incorporates another compound's lighter ^{12}C peak start or heavier ^{13}C peak tail (Meier-Augenstein, 2002; Sessions, 2006). Since derivatization adds external carbon to the AAs, ^{13}C-AA data require correction using mass-balance equations and predefined isotope correction factors (Figure 7: Step 10, Docherty et al., 2001). Long-term measurement stability and instrument performance are monitored through regular analysis of external reference compounds with known isotope values (Figure 7: Step 11).

Pitfalls in the analytical workflow

Sample pre-treatments and purification protocols vary widely in their complexity and scope (Figure 7: Steps 1, 4, 5),

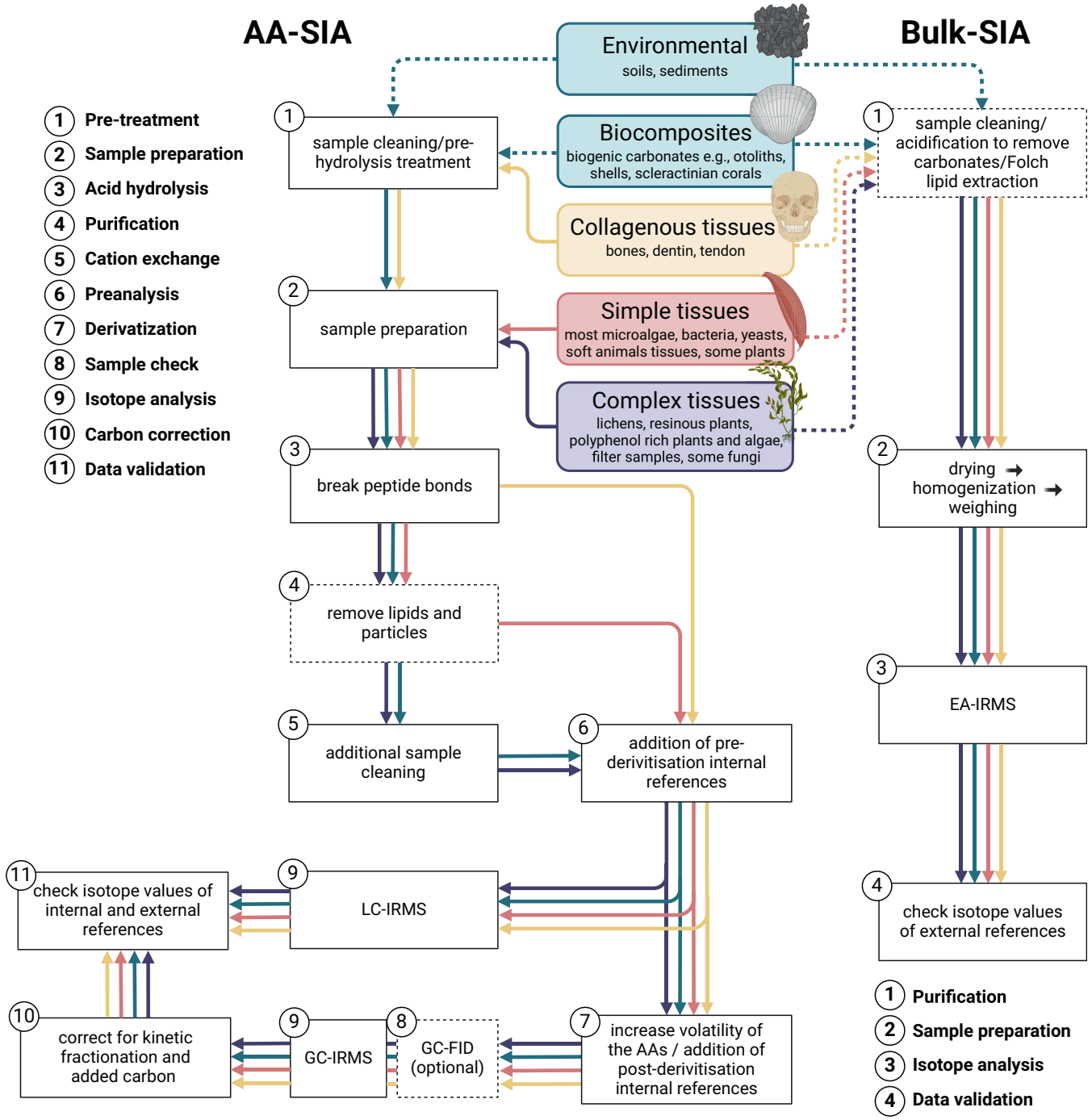

FIGURE 7 Analytical protocol workflows for proteinogenic amino acid (AA) stable isotope analysis (SIA) of different sample types (colored boxes and corresponding arrows) measured with liquid or gas chromatography isotope mass spectrometry (LC-IRMS or GC-IRMS) contrasted against bulk stable isotope analysis with an elemental analyzer isotope ratio mass spectrometer (EA-IRMS). Gas chromatography with flame ionization detection (GC-FID) is optional to assess AA concentrations in a sample. Broken line arrows and boxes indicate that the treatment step is not mandatory for all samples. The illustration was created with BioRender.com.

yet their potential bias on δ^{13}C-AA values remains largely unexplored. Although acidic treatments and chemical extractions with extensive water rinsing are commonly used to remove minerals, urea, and lipids from consumer tissues, these methods are discouraged as they lead to large and inconsistent bulk isotopic measurements (Brodie et al., 2011; Huang et al., 2023; Pellegrini & Snoeck, 2016; Schlacher & Connolly, 2014). Such aqueous pretreatments may result in AA loss and alter δ^{13}C-EAA values in consumer tissues relative to untreated dietary tissues (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 utilizing extensive aqueous 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 this observation was based on recurring methodological patterns, we call for more studies on pretreatment protocols and methodology in general. Demonstrably less bias prone are post-hydrolysis purification methods, such as cation exchange or solid-phase extractions (McMahon et al., 2010; Ohkouchi et al., 2017; Takano et al., 2010). For carbonate samples, acid hydrolysis converts calcium carbonate to calcium chloride, a compound that readily absorbs water. Water-sensitive derivatization reagents, such as acetyl chloride and acetic anhydride, react with water and form compounds that can co-elute with the AAs during chromatography. This can be mitigated by using post-hydrolysis purification with cation exchange resins, or using water-insensitive reagents (e.g., methoxycarbonyl esterification [MOC], Vane et al., 2018; Walsh et al., 2014).

Correcting for exogenous carbon addition during AA derivatization remains challenging (Figure 7: Step 10, Docherty et al., 2001; Takizawa et al., 2020). Using derivatization reagents with δ^{13}C values similar to sample values and ensuring complete reactions helps minimizing errors. Methods like MOC and N-acetyl methylation (NACME) introduce minimal additional carbon and produce stable derivatized AAs suitable for long-term storage, offering advantages over the trifluoroacetic acid anhydride (TFAA) method (Corr et al., 2007; Walsh et al., 2014). Different derivatization methods, such as MOC, NACME, and TFAA, can introduce biases in individual δ^{13}C-AA values, making direct comparisons impractical (Dunn et al., 2011; Walsh et al., 2014). Sample drying during and after derivatization requires careful monitoring, as excessive drying can cause partial evaporation of low-molecular-weight AAs.

The need for standardization

Maintaining δ^{13}C-AA measurement integrity requires careful monitoring of analytical consistency and stability over time. Complete peak separation and Gaussian peak shapes are fundamental for accurate isotopic measurements (Meier-Augenstein, 2002; Sessions, 2006). Monitoring instrument stability and accuracy, that is, measurement standardization, is achieved with internal and external reference compounds (Meier-Augenstein & Schimmelmann, 2019). Internal references are added directly to the sample (Figure 6: Steps 6 and 7) to provide calibration, track isotope effects, and monitor AA losses. To calculate appropriate concentrations of internal references, sample AA concentrations can be determined using GC with flame ionization detection (GC-FID, Figure 7: Step 8). External references are measured separately to serve as benchmarks (Figure 6: Step 11) and should encompass the range of δ^{13}C-AA values in the samples. References can be subdivided into two categories: derivatized AAs with known pre-derivatization δ^{13}C values (Meier-Augenstein & Schimmelmann, 2019; Roberts et al., 2018) and non-derivatized compounds, for example, 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 δ^{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 the practice of thoroughly detailing their methodological protocols in publications, as proposed by Dunn and Skrzypek (2023). To that end, the research community should share and analyze a common set of relevant biological reference materials. This aligns with broader initiatives in isotope analysis that call for standardized reference materials for direct data comparisons between laboratories (e.g., de Laeter, 2005; Gröning, 2004; Stichler, 1995). Selecting suitable references for δ^{13}C-AA analysis requires materials that are homogeneous, easily transportable, and neither hazardous nor biologically active. International reference materials meeting these criteria would strengthen the reliability and comparability of δ^{13}C-AA measurements across laboratories. Standardizing 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 δ^{13}C-EAA fingerprint library (*Applying δ^{13}C-EAA fingerprints in ecological studies*).

FROM QUALIFYING TO QUANTIFYING BASAL RESOURCE USE

Consumer tissue δ^{13}C-EAA patterns are a composition of the δ^{13}C-EAA patterns of the assimilated basal organisms. Compositional data analysis has a long history,

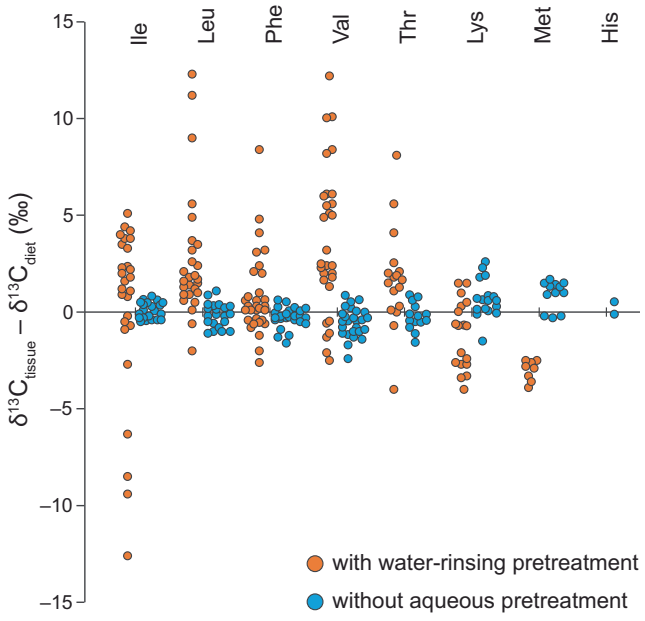

FIGURE 8 The differences in measured $\delta^{13}C$ values for individual EAAs, isoleucine (Ile), leucine (Leu), phenylalanine (Phe), valine (Val), threonine (Thr), lysine (Lys), methionine (Met), histidine (His), observed between diet and consumer tissue in 17 controlled feeding studies divided on the use of water-rinsing or soaking 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 between different diets (e.g., protein origin, macronutrient composition, or prey organisms). See Appendix S5 for specific details on individual studies.

spanning from geology to remote sensing (Aitchison, 1982; Clevers & Zurita-Milla, 2008; Weltje, 1997). The statistical frameworks used to estimate proportional contributions in compositional data are linear (un)mixing models (Parnell et al., 2013; Phillips, 2012; Weltje, 1997). Over the past 20 years, significant development of mixing models has addressed many issues associated with biological systems, including complex data structures (Semmens et al., 2009; Stock et al., 2018), under-determined mixing systems where many potential basal organism combinations could result in the same $\delta^{13}C$-EAA patterns (Parnell et al., 2010), and incorporating natural variations and measurement uncertainties (Moore & Semmens, 2008; Stock et al., 2018). Here we outline the application of mixing models with $\delta^{13}C$-EAA data, highlighting key considerations, assumptions, and limitations (but see Cheung & Szpak, 2021; Phillips et al., 2014; Stock et al., 2018 for general reviews of best practices). While several implementations of mixing models are available, we primarily focus on the MixSIAR package in *R* (Stock et al., 2018) due to its flexibility and relatively common use across ecology (but see Wang, Lu, & Fu, 2019, and Cheung & Szpak, 2021 for direct software comparisons).

Consolidating basal organism information

The area bounded by basal organism $\delta^{13}C$-EAA fingerprints, the endmembers, constitutes the mixing space: the area containing all possible consumer tissue $\delta^{13}C$-EAA patterns (Phillips et al., 2014; Smith et al., 2013). The mixing space dimensionality is equal to the number of EAAs measured, the mixing model tracers. All basal organisms that likely contribute to consumer $\delta^{13}C$-EAA patterns should be characterized (see *Considerations for the $\delta^{13}C$-EAA patterns of facultative prototrophs*), as proportional contributions of basal resources are not independent of each other: they must, by definition, sum to one. When resolving mixing systems, missing endmembers is a general problem (Weltje, 1997), resulting in inaccurate proportions regardless of the statistical approach. Consumer $\delta^{13}C$-EAA data falling outside of the mixing space can indicate missing basal organism groups, although consumers falling within the mixing space may still utilize basal resources that have not been characterized. Conversely, it is important to limit basal organisms to only those that likely contribute to consumers. It may seem reasonable to include as many basal organisms as possible, but a key assumption of mixing models is that all included basal organisms contribute to the consumer $\delta^{13}C$-EAA values to some degree. Excluding unused basal organisms limits model complexity, aiding model performance, and improves model accuracy by removing isotopically feasible but biologically unrealistic combinations. Further, statistical artifacts arise when resolving mixing models with high numbers of basal organisms as solutions will tend toward $1/n$ for large n: it is recommended to limit mixing models to seven or fewer endmembers (Stock et al., 2018).

Trophic discrimination factors (TDFs, Table 1) need to be quantified for many types of tracers (Schulting et al., 2022), but they are negligible for $\delta^{13}C$-EAA data (e.g., McMahon et al., 2010, Figure 8, Appendix S6). However, the natural variation in basal organism $\delta^{13}C$-EAA values needs careful consideration as it can be inadequately described when logistical and analytical constraints result in low sample sizes. While low sample sizes can be accounted for in Bayesian mixing models, it reduces model precision. $\delta^{13}C$-EAA variability could be approximated using well-constrained literature sources; however, differences in methodologies and analytical processes can add additional uncertainties in $\delta^{13}C$-EAA data (See *Minimizing analytical uncertainties in $\delta^{13}C$-AA value*). Analytical precision is rarely considered when

quantifying basal resource use with δ^{13}C-EAA data (Hopkins & 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 (see Appendix S6).

Modeling consumer behavior

Specific hypotheses regarding consumer basal resource use inform how mixing models are structured. Different nutritional requirements or access to specific dietary items or habitats that can vary with factors such as species, sex, size or ontogenetic stage, or social status may result in differences in basal resource use between consumers. Hierarchical spatial structuring of consumers, such as distinct subpopulations within larger areas or spatially discrete sampling sites, should be considered as spatial structuring can affect basal organism availability and use, even if preferences are the same among individual consumers (Semmens et al., 2009). This similarly applies to consumers sampled in different time periods (e.g., seasons, years).

Bayesian mixing models can incorporate prior information to inform model solutions, such as estimates extracted from mass-balanced food web models (Stock et al., 2018). However, prior information can be biased (e.g., stomach and scat data toward poorly digestible prey), and may overly restrict mixing model solutions (Swan et al., 2020, but see Brown et al., 2018). Theoretically, known nutritional limitations such as macronutrient requirements can be included as prior information where consumers have considerable diversity in diet quality, for example, in FRUITS mixing model software (Fernandes et al., 2014). However, as prior information typically pertains to consumer diet (i.e., the proportions of prey ingested) rather than basal resource use, it 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 modeled as a multiplicative term called a "residual stretch error," rather than as a normal distribution, that stretches or compresses the variance attributed to model processes (stochastic sampling of basal resource variation and additional uncertainties, Stock & Semmens, 2016; Stock et al., 2018). The ecological justification for the residual stretch error approach is that consumers feed many times, dampening the isotopic variation observed in basal organisms. This contrasts with the implementation of mixing models that sample basal organism δ^{13}C-EAA values from their distributions only once when estimating model solutions (Stock & Semmens, 2016). Residual stretch errors should therefore take values between 0 and 1 to compress variation due to feeding behaviors. Values approaching zero can be interpreted as an increase in the number of feeding events reflected in the consumer tissue, whereas values greater than 1 indicate that factors beyond those included in the mixing model contribute to individual variation. For passive trophic behaviors such as sessile filter-feeding or grazing, the stretch error approach works well (Stock & Semmens, 2016). However, active and selective feeding modes in motile consumers may violate the assumption of stochastic sampling, inflating residual stretch errors, where it may be more appropriate to incorporate individuals as an additional random effect in the model structure. The drawback however 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 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 performance (Cheung & Szpak, 2021), emphasis should be given to the biological interpretations and their trade-offs within the studied system.

Interpreting mixing model output

Underpinning a mixing model's ability to accurately estimate basal resource use by the consumer is the separation in δ^{13}C-EAA data between basal organisms. 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 two basal organism δ^{13}C-EAA patterns cannot be distinguished, that is, are not δ^{13}C-EAA fingerprints, then their posterior contributions will be negatively correlated, and potentially exhibit bimodality (Phillips et al., 2014). In such cases, the proportional contributions of the two groups should be combined into a single group post analysis, which often drastically reduces overall uncertainty (Phillips et al., 2014). Similarity between δ^{13}C-EAA patterns is often tested statistically by comparing the mean δ^{13}C-EAA offsets for each AA separately. However, equality of means testing depends on large endmember sample sizes to be robust (Stock et al., 2018), which is typically not the case for δ^{13}C-EAA measurements, and does not consider differences in variances and covariances between basal organisms. If required, statistical

scrutiny should be conducted using a multivariate approach (e.g., Bhattacharyya coefficients, Figure 4). If two or more basal organism groups 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). Similarly, if all basal organisms exhibit similar means across one or more EAAs, then it may seem logical to remove such tracers to reduce model complexity and aid model convergence; however, differences in basal resource (co)variances also contribute to resolving mixing models. As demonstrated for δ^{13}C-EAA data from controlled feeding experiments, mixing models including all measured EAAs provide the most accurate solutions across diets with reduced uncertainties compared with those using a restricted set of AAs or other statistical approaches (Manlick & Newsome, 2022); therefore, users should be cautious about arbitrarily removing tracers.

When implemented, residual stretch errors identify insufficiently characterized basal organism groups and highlight potential issues with model components, for example, analytical uncertainty. Stretch errors slightly greater than 1 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 1 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 missing basal organism clades that significantly differ in δ^{13}C values for the identified EAAs (Vane et al., 2023, Appendix S6) 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 organisms and consumers within it, and their uncertainties. Precisely quantifying basal resource use can therefore be highly ecosystem specific. If consumers depend on only a few, isotopically similar basal organisms, 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 often reflected in large uncertainties in mixing model solutions, capturing the true uncertainty associated with disentangling basal resource use. This can be verified by quantifying how informative the input data have been in updating the mixing model priors (Brown et al., 2018). In such instances, using baseline δ^{13}C-EAA values may prove fruitful where strong environmental gradients separate basal organisms, but comes with greater logistical restraints such as in situ sampling (Vane et al., 2023, see *Beyond δ^{13}C-EAA fingerprints*).

Considerations when quantifying basal resource use

Quantitative approaches using δ^{13}C-EAA data provide complementary benefits, but additional complexities compared with bulk stable isotope data (See *Minimizing analytical uncertainties in δ^{13}C-AA value*), notably the logistical difficulties in adequately characterizing all basal organisms in situ. This has likely led to the application of extensive training datasets becoming commonplace in δ^{13}C-EAA studies (e.g., Arsenault, Thorp, et al., 2022; Arthur et al., 2014; Macartney et al., 2020; Rowe et al., 2019, see *Applying δ^{13}C-EAA fingerprints in ecological studies*). However, such training datasets can result in inflated variation and potential bias (mean offsets) in δ^{13}C-EAA patterns, preventing the characterization of δ^{13}C-EAA fingerprints compared with in situ sampling (See *Applying δ^{13}C-EAA fingerprints in ecological studies* and *Pitfalls in the analytical workflow*). These issues may give rise to conflicting and/or inaccurate inferences depending on the approach taken. We illustrate this in Figure 9 where we show the variability in a global training dataset compared with ecosystem specific but limited in-study sampling of basal organisms in LDA space. Mean δ^{13}C-EAA pattern bias between the two datasets can be observed for several groups, notably fungi, and inflated variation means that study-specific δ^{13}C-EAA fingerprints are lost when using global data compilations. Mixing models are sensitive to input data (Bond & Diamond, 2011); therefore, the contrasting basal organism datasets lead to apparent differences in basal resource use, as shown in Figure 9b,c. The model with global training data suggests higher microalgal EAA contributions to *Daphnia* in oligotrophic Arctic lakes compared with data measured in situ (see Appendix S6 for a more detailed contrast and discussion on the two approaches). In some instances, logistical constraints limit complete basal organism characterization in situ, necessitating the use of carefully selected external training data. If incorporated,

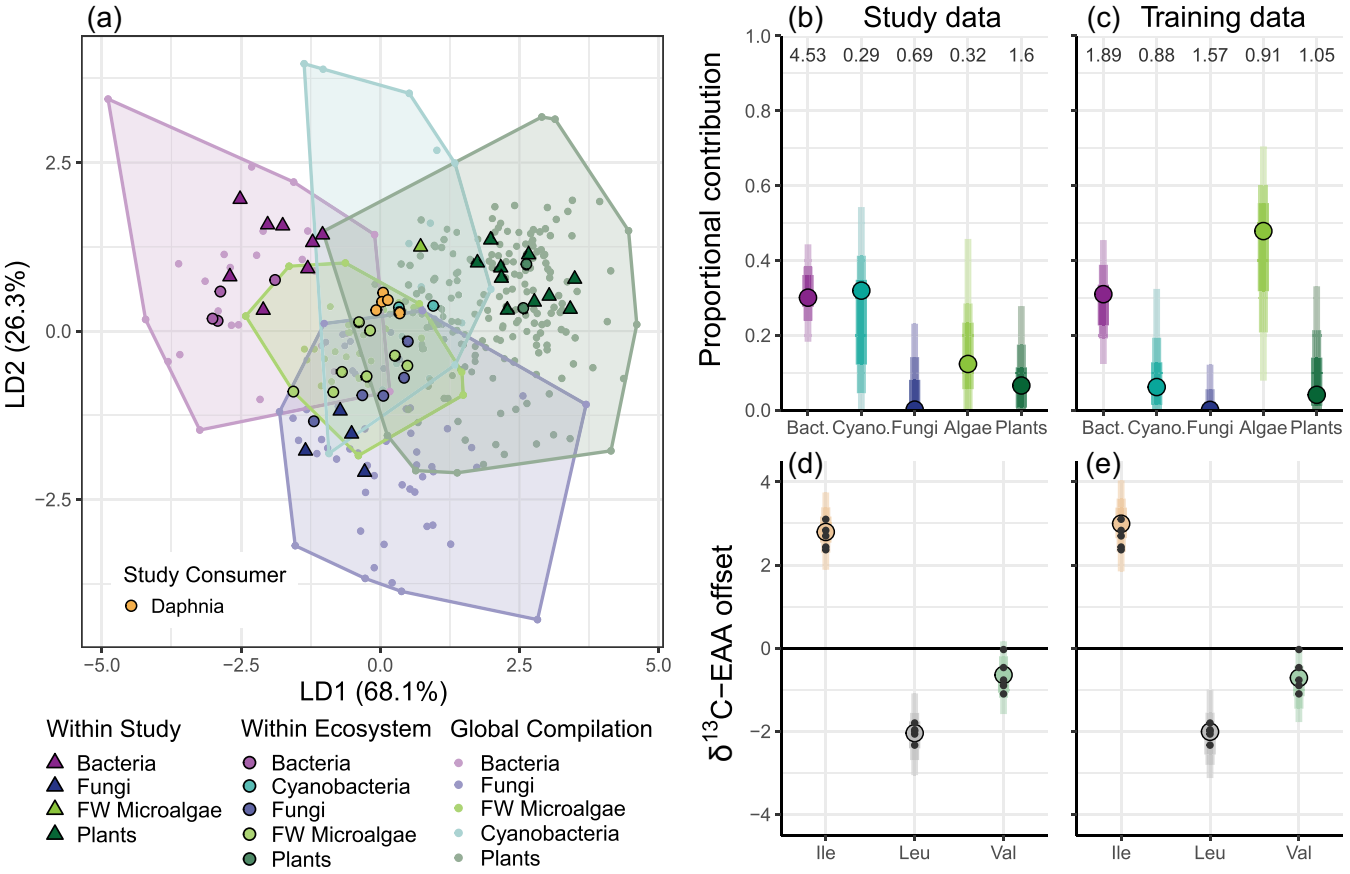

FIGURE 9 Linear discriminant analysis highlighting the increased variation and mean bias introduced to δ^{13}C-EAA patterns in basal organisms when using training data (individual data points plus their convex hulls, Figure 4) compared with in situ sampling for quantifying basal resource use. Study data are from Larsen et al. (2013) examining basal resource use by *Daphnia* sp. in oligotrophic lakes in Alaska. Plot (a) shows an LDA of the δ^{13}C-EAA patterns (Leu, leucine; Ile, Isoleucine; Phe, phenylalanine; Thr, threonine; Val, valine) in the five main basal organism groups measured within the study considered relevant to *Daphnia* sp. compared with using global training data. Study data consist of samples taken directly from the Alaskan lake ecosystems, within-study, plotted as filled triangles, and organisms from cultures or sampled from other cold-water lake ecosystems and forests, within-ecosystem, are plotted as filled circles. Training data for these basal organism groups are taken from the global compilation in *Discriminating basal organisms with δ^{13}C-EAA fingerprints* section, plotted as colored dots (see Appendix S6: Table S1). The within-study freshwater (FW) microalgae consists of a single seston filtratecomposite, which is likely a mixture of microalgae and allochtonous POM, falls outside of the cultured data, and therefore not included within in situ sampling. Mixing model contributions when using study data, plot (b), and training data, plot (c), highlight the bias that can occur when using training data. How informative basal organism group data are to mixing model outputs is shown at the top of plots B and C, quantified as the bootstrapped median Kullback–Leibler (KL) divergence of the marginal contributions (see Appendix S6 for details). Local study data provide strong evidence for bacterial proportions (high KL value), but limited evidence for the estimated proportions of cyanobacteria and microalgae (low KL). Global training data however reasonably inform the model across all basal organisms. Both setups do equally well at reconstructing δ^{13}C-EAA patterns in *Daphnia* sp., see plots (d) versus (e), highlighting that this is a poor method for comparative mixing model validation (Brown et al., 2018). Posterior credible intervals are plotted as 50%, 75%, and 95% highest probability density intervals as bars of decreasing thickness and color saturation, with posterior modes plotted as filled circles. Observed δ^{13}C-EAA offsets in *Daphnia* sp. are plotted as black dots in (d) and (e).

training data should have clearly documented metadata, align with analytical protocols (e.g., use the same derivatization methods), and, ideally, inter-laboratory analytical variability is accounted for. As international reference materials are currently lacking, external data should generally not be used as a substitute for adequate system sampling if the aim is to accurately quantify basal resource use and differences therein, especially at local scales. Currently, we would advise adequate sampling of basal organisms with each new study where possible.

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

(Fox et al., 2019). Arguments for this approach include a "less rigid framework" regarding uncharacterized basal organisms and mixing space geometry (Fox et al., 2019; Manlick & Newsome, 2022). Such arguments misconstrue that the "rigid" assumptions are inherent to the statistics rather than the 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, as exemplified in Figure 9a where *Daphnia* δ^{13}C-EAA patterns appear to be encapsulated by the basal organisms, but in fact fall outside the mixing space for some EAAs (Appendix S6, see also Appendix S4). Such observations are more noticeable when implementing Bayesian mixing models as EAA data are often directly visualized, or are implied by exceedingly large stretch errors. As LDA approaches classify data rather than reconstructing mixtures, they are highly sensitive to data geometry and therefore can readily produce unreliable results dominated by a single basal organism group (Manlick & Newsome, 2022; Skinner et al., 2021, and see Appendix S4). In fact, as consumer δ^{13}C-EAA patterns are a mixture, rather than wholly one of the defined basal organism groups, the main assumption of LDA classification is violated a priori. Recent simulations on lake ecosystem data highlight that significant bias can occur between known basal resource contributions and those estimated using this LDA bootstrapping approach (Saboret et al., 2023). As the LDA bootstrapping procedures only influence the position of decision boundaries, this does not truly incorporate uncertainty in the same way as mixing models. Instead, it only affects classifications of consumer data falling relatively equidistant between basal organism groups. As LDA minimizes intragroup variability, uncertainty estimates are artificially deflated giving a false view of precision. This not only influences LDA bootstrapping but may also be problematic if practitioners use LD coordinates in mixing models rather than δ^{13}C-EAA values. 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 consumers (Makarewicz & Sealy, 2015). This can be incorporated directly into Bayesian mixing models through prior information and the rich and diverse model structures, which is simply not achievable with LDA boot-strapping approaches. It is frequently highlighted that mixing models are only as good as the input data (Phillips et al., 2014), yet quantifying basal resource use is also only as good as the mathematical abstraction used to describe our understanding of ecosystem processes.

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 into including 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 (See *The diagnostic potential of δ^{13}C-EAA patterns among basal organisms*, Elliott Smith et al., 2022; Larsen et al., 2020; Scott et al., 2006; Stahl et al., 2023; Vane 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 *Factors shaping amino acid δ^{13}C values in basal organisms* and *Discriminating basal organisms with δ^{13}C-EAA fingerprints*, would facilitate targeted analyses of discriminatory resolution. It is important to note that δ^{13}C-EAA patterns may not reflect ecological distinctions in basal organisms. For example, it can be challenging discerning between fresh tissues and their detrital material, because δ^{13}C-EAA patterns remain relatively consistent during tissue necrosis, fragmentation, and detrital transport (Elliott Smith et al., 2022; Larsen et al., 2013; Vane et al., 2023).

Extending beyond discriminating basal organisms and reconstructing basal resource use in consumers, extracting the full extent 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. Research during the last decade has advanced our understanding of the mechanisms underpinning δ^{13}C-AA data (e.g., Elliott Smith et al., 2022; Larsen et al., 2015; Manlick & Newsome, 2022; Stahl et al., 2023). However, the rapid increase in the use of these data in ecological research highlights the need for further integrating and expanding mechanistic insights for full comprehensive analyses. For instance, new physiological hypotheses regarding basal organisms can be generated through δ^{13}C-AA data, such as the synthesis of ^{13}C-deplete lignin resulting in relatively enriched δ^{13}C values of phenylalanine in vascular plants (See *The diagnostic potential of δ^{13}C-EAA patterns among basal organisms*). 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 synthesized AAs, which can be compared with 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 further facilitated by the development of position-specific stable isotope analyses (Fry et al., 2023).

Despite the richness of information that δ^{13}C-AA data provides, the large analytical effort has likely contributed to the trend of incorporating external training data into study designs, varying from graphical comparisons (e.g., Besser et al., 2022; Larsen et al., 2012; Stahl et al., 2023) to extensive training data within mixing models (e.g., Arsenault, Thorp, et al., 2022; Arthur et al., 2014; Rowe et al., 2019). 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., de Laeter, 2005; Gröning, 2004; Stichler, 1995). Direct δ^{13}C-AA data comparisons should be facilitated by international reference materials and the standardization of analytical methodologies across research facilities (Figure 7). Studies comparing inter-lab methodologies would 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 training data into study designs. Ultimately, δ^{13}C-AA values of basal organisms could be collated into a single taxa-specific reference library for future studies, constituting a separate functional role to the wider calls for a centralized 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 fingerprint approach affords the opportunity to explore carbon fluxes across spatiotemporal scales without having to characterize changes in baseline δ^{13}C-EAA values, offering basal organism characterization and tracing that is unparalleled in its specificity and inclusivity. Understanding basal resource use by metazoans and their physiological responses in conjunction with changes in basal organism abundance, composition, nutritional quality, and the environment provides valuable insights into the resilience of differing food webs across the world.

AUTHOR CONTRIBUTIONS

All authors contributed equally to this manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

Data (Cobain, Larsen & Vane, 2023) are available in Figshare at https://doi.org/10.6084/m9.figshare.22852355.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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