

RESEARCH ARTICLE

Assessing the potential of amino acid $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ analysis in terrestrial and freshwater ecosystems

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Abstract

1. Understanding the structure and dynamics of food webs requires accurate estimates of energy flow among organisms. Bulk tissue carbon ($\delta^{13}\text{C}$) and nitrogen ($\delta^{15}\text{N}$) isotope analysis is often used to this end; however, the limitations of this technique can outweigh the benefits. The isotope analysis of individual amino acids is being increasingly employed to trace energy flow and estimate consumer trophic level. Central to this compound-specific approach are the concepts of essential amino acid (AA_{ESS}) $\delta^{13}\text{C}$ fingerprinting and amino acid (AA) $\delta^{15}\text{N}$ β -values, both of which have been understudied and are poorly constrained in terrestrial and freshwater producers.
2. We present AA_{ESS} $\delta^{13}\text{C}$ data for 112 terrestrial and freshwater producers collected from two aridland habitats in the northern Chihuahuan Desert (New Mexico, USA) and AA $\delta^{15}\text{N}$ data for a subset ($n = 28$) of these samples. We characterized AA_{ESS} $\delta^{13}\text{C}$ fingerprints by performing linear discriminant analysis on the $\delta^{13}\text{C}$ values of isoleucine, leucine, lysine, phenylalanine, threonine and valine for four producer groups— C_3 plants, C_4 plants, CAM plants and filamentous green algae. We explored potential biochemical mechanisms underlying these AA_{ESS} $\delta^{13}\text{C}$ fingerprints by calculating differences between the $\delta^{13}\text{C}$ values of AA_{ESS} products and their AA precursors. This allowed us to estimate and compare isotopic discrimination for specific AA_{ESS} synthesis pathways across producer groups.
3. We found a near-perfect separation of AA_{ESS} $\delta^{13}\text{C}$ fingerprints among producer groups; all groups reclassified with >95% success within our multivariate framework. We also found varied isotopic discrimination for specific AA_{ESS} synthesis pathways among producer groups. Contrary to previous studies, we found no differences in β -values between terrestrial C_3 and C_4 plants for any trophic-source AA pairing. Furthermore, we found that Lys $\delta^{15}\text{N}$ values were less variable and more closely related to bulk tissue $\delta^{15}\text{N}$ values than Phe $\delta^{15}\text{N}$ values in terrestrial and freshwater producers.
4. *Synthesis.* We conclude that AA_{ESS} $\delta^{13}\text{C}$ fingerprints are a higher resolution tracer for freshwater food webs, where instream algae have overlapping bulk tissue $\delta^{13}\text{C}$ values with terrestrial C_3 plants. Additionally, $\beta_{\text{Glx-Lys}}$ and $\beta_{\text{Pro-Lys}}$ are

the best for AA $\delta^{15}\text{N}$ -based consumer trophic-level estimates in freshwater food webs containing both terrestrial and aquatic resources.

KEYWORDS

algae, β -values, carbon isotope fingerprinting, essential amino acids, food webs, nitrogen, plants, trophic and source amino acids

1 | INTRODUCTION

The use of carbon ($\delta^{13}\text{C}$) and nitrogen ($\delta^{15}\text{N}$) amino acid (AA) isotope analysis in food web studies has grown in popularity over the past couple decades. Although AA isotope analysis is substantially more expensive and labour-intensive than bulk tissue isotope analysis, it provides an order of magnitude more data points per sample (i.e. δ -values for 12–14 AAs), allows for greater isotopic separation among primary producer groups, may not require baseline sampling in certain cases and can provide deeper insights into producer and consumer physiology. Despite the increasing number of studies that have utilized this promising compound-specific approach to examine consumer trophic ecology and energy flow in food webs, AA $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ patterns in primary producers are comparatively understudied, particularly in terrestrial and freshwater ecosystems. Additionally, most studies examining primary producer AA $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values have focused on data from only a subset of two to six AAs. Given this and the limitations of bulk tissue and other compound-specific isotope analyses in these systems, the AA $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ patterns in terrestrial and freshwater producers warrant further study.

The $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ analysis of bulk tissues is often used to trace the relative importance of different sources of primary production (Reid et al., 2008), estimate food chain length (Cabana & Rasmussen, 1996) and quantify food web structure (Layman et al., 2012). Despite its widespread utility, this approach has a number of limitations (Whiteman et al., 2019). The first, and often most apparent, limitation is that the $\delta^{13}\text{C}$ values of distinct energy channels often overlap. For example, $\delta^{13}\text{C}$ analysis is often used to trace terrestrial (allochthonous) and instream (autochthonous) resources supporting freshwater consumers (Finlay, 2001; Grey et al., 2001; Tanentzap et al., 2017); however, the $\delta^{13}\text{C}$ values of C_3 plants and freshwater algae collected from the same locality can be similar (Finlay et al., 2010; Finlay & Kendall, 2007). Such overlap necessitates careful characterization of the 'baseline' (i.e. primary producer) isotopic composition of food webs, which can vary considerably across space and time (Cabana & Rasmussen, 1996; Hershey et al., 2017; Turner et al., 2015). Another limitation of bulk tissue isotope analysis is the need to apply trophic discrimination factors (TDFs) that account for physiologically driven offsets in the isotopic composition of consumer tissue relative to that of potential dietary items. TDFs for both $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ can vary substantially across taxa, tissue types and dietary protein content or quality (Caut et al., 2009). Overall, these factors make it difficult to quantify the

relative importance of distinct energy channels in fuelling food webs and to estimate the trophic level (TL) of organisms within those food webs.

Compound-specific stable isotope analysis (CSIA) of individual AAs or fatty acids (FAs) continues to grow as a robust proxy for tracing energy sources and estimating consumer TL. For example, FA $\delta^2\text{H}$ and $\delta^{13}\text{C}$ values are promising tracers for food web studies (Anparasan & Hobson, 2021; Budge et al., 2011; Burian et al., 2020; Chiapella et al., 2021; Wang et al., 2015), especially those in which consumers rely on both terrestrial and aquatic resources. Aquatic algae and terrestrial plants typically have distinct FA profiles, with algae containing higher concentrations of polyunsaturated fatty acids (PUFAs; Gladyshev et al., 2013; Hixson et al., 2015). Thus, FA profiles can be combined with FA $\delta^2\text{H}$ and/or $\delta^{13}\text{C}$ analysis to trace dietary sources in animals (Twining et al., 2020). Arguably, studying food webs in terms of essential and conditionally essential biomolecules may be more relevant for assessing consumer nutrition, important trophic interactions and overall ecosystem health and functioning than studying them in terms of energy in units of carbon or biomass (Ruess & Müller-Navarra, 2019). While essential FAs and PUFAs are vital for animal health and reproduction (Rivers et al., 1975; Twining et al., 2016), AAs are the major macromolecular constituents of animal tissues. Recent advances in AA isotope analysis show great promise in overcoming the limitations associated with the isotopic analysis of bulk tissues (Whiteman et al., 2019).

Organisms that can synthesize essential amino acids (AA_{ESS}) *de novo* use a variety of biochemical pathways and enzymes to do so, which imprints on their AA_{ESS} $\delta^{13}\text{C}$ values to create distinct isotopic 'fingerprints' that differ among plants, marine and freshwater algae, fungi and bacteria (Elliott Smith et al., 2018; Larsen et al., 2009, 2013; McMahan et al., 2016; Pollierer et al., 2020; Scott et al., 2006). Since animals cannot synthesize AA_{ESS} *de novo* and must acquire them from their diet, these compounds move through the food web virtually unaltered isotopically (McMahon et al., 2015), eliminating the need to account for trophic discrimination when tracing energy sources; however, see Newsome et al. (2011, 2020) for evidence the gut microbiome may supply AA_{ESS} to protein-limited hosts. Despite the great potential of this approach, only a handful of studies have tested the efficacy of AA_{ESS} $\delta^{13}\text{C}$ fingerprinting in distinguishing among co-occurring producer groups (Elliott Smith et al., 2018; Larsen et al., 2009, 2012; McMahan et al., 2016; Pollierer et al., 2020), and no study has sufficiently characterized terrestrial and freshwater producer

AA_{ESS} $\delta^{13}\text{C}$ fingerprints at the landscape scale with large sample sizes of wild-collected plants and algae (but see Larsen et al., 2016 for co-occurring grass and clover leaf and root AA_{ESS} $\delta^{13}\text{C}$ fingerprints). Additionally, no study has quantified differences in AA_{ESS} $\delta^{13}\text{C}$ fingerprints among wild terrestrial plants that use different photosynthetic pathways (C_3 , C_4 and CAM).

The $\delta^{15}\text{N}$ analysis of individual AAs likewise holds promise for estimating consumer TL in terrestrial and freshwater food webs. Unlike the traditional biochemical classifications used for $\delta^{13}\text{C}$ analysis, AAs have been empirically classified as 'source' or 'trophic' in the context of $\delta^{15}\text{N}$ analysis (Popp et al., 2007), reflecting the relative degree of transamination they experience during metabolism (O'Connell, 2017). $\delta^{15}\text{N}$ values of source AAs (e.g. phenylalanine and lysine) remain relatively unchanged as they move up food chains, whereas $\delta^{15}\text{N}$ values of trophic AAs (e.g. glutamic acid and proline) increase by ~5–8‰ per trophic step (McMahon & McCarthy, 2016; but see L ubcker et al., 2020 for influences of fasting and Shipley et al., in revision for influences of pregnancy on AA $\delta^{15}\text{N}$ values). Many studies have utilized these patterns to calculate consumer TL using the equation: $\text{TL} = 1 + [(\delta^{15}\text{N}_{\text{ConsumerTrophicAA}} - \delta^{15}\text{N}_{\text{ConsumerSourceAA}} - \beta)/\text{TDF}]$, where $\beta = \delta^{15}\text{N}_{\text{TrophicAA}} - \delta^{15}\text{N}_{\text{SourceAA}}$ in local primary producers and $\text{TDF} = \Delta^{15}\text{N}_{\text{TrophicAA}} - \Delta^{15}\text{N}_{\text{SourceAA}}$ between the consumer and its diet (Chikaraishi et al., 2009). This approach provides TL estimates from a single consumer tissue sample without the need for baseline $\delta^{15}\text{N}$ data (Matthews et al., 2020; McMahon & McCarthy, 2016). However, these TL estimates are dependent on accurate β -values, which are currently poorly constrained in terrestrial and freshwater primary producers (but see Ishikawa et al., 2014; Kendall et al., 2019). Furthermore, a recent meta-analysis recommended collecting representative primary producers from focal food webs to better constrain β -values (Ramirez et al., 2021), and no study has measured AA $\delta^{15}\text{N}$ values and β -values in wild C_3 , C_4 and CAM plants collected from the same location.

Our goal was to develop a landscape-scale library of AA_{ESS} $\delta^{13}\text{C}$ fingerprints for freshwater filamentous green algae and terrestrial plants from two aridland habitats in the northern Chihuahuan Desert (New Mexico, USA). We quantified variation in AA_{ESS} $\delta^{13}\text{C}$ fingerprints among producer groups to identify the utility of this technique for studying energy flow, ecosystem connectivity and food web structure in these ecosystems. For a subset of these samples, we also measured AA $\delta^{15}\text{N}$ values and examined variation in β -values among producer groups. Using a biochemical framework, we explored potential mechanisms for the variation we observed in AA $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values and patterns across producer groups. This study represents an important advancement in the application of AA-based isotope approaches to terrestrial and freshwater ecosystem studies because it is the first to comprehensively describe patterns among terrestrial and freshwater primary producers at a landscape scale, a necessary step in utilizing these cutting-edge approaches to characterize energy flow within and among these two types of habitats.

2 | MATERIALS AND METHODS

2.1 | Study sites

Filamentous green algae samples were collected in September 2015 and March 2018 from the middle Rio Grande near Algodones, NM, USA. Plant samples were collected in June and September of 2017 and 2018 from two localities: (a) the riparian gallery forest (Bosque) along the middle Rio Grande near Albuquerque, NM, USA and (b) mixed shrubland–grassland habitat in the northern Chihuahuan Desert at the Sevilleta National Wildlife Refuge in Socorro County, NM, USA.

2.2 | Bulk tissue $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ sample preparation and analysis

Approximately 5 mg of dried plant leaves or seeds and filamentous green algae were packed into $3 \times 5\text{ mm}$ tin capsules. $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values were measured using a Costech 4010 elemental analyser connected to a Thermo Scientific Delta V Plus isotope ratio mass spectrometer at the University of New Mexico Center for Stable Isotopes (UNM-CSI; Albuquerque, NM). Isotope values are expressed in delta (δ) notation (Equation S1). Analytical precision was measured as mean within-run standard deviations (SD) of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values of an in-house reference material (*Aesculus californica*); mean within-run SD were all <0.2‰. Weight per cent carbon and nitrogen concentrations, reported as C:N ratios, were also measured as a means of estimating protein content (Vrede et al., 2004).

2.3 | Amino acid $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ sample preparation and analysis

Approximately 10–20 mg of producer tissues were hydrolysed, purified and derivatized to *N*-trifluoroacetic acid isopropyl esters alongside an in-house AA reference material containing a mixture of commercially available AA powders of known isotopic composition following established protocols (Protocol S1; Table S1; Amelung & Zhang, 2001; Silfer et al., 1991). Note that hydrolysis converts glutamine into glutamic acid and asparagine into aspartic acid. We reliably measured the $\delta^{13}\text{C}$ values of six AAs considered essential for animals— isoleucine (Ile), leucine (Leu), lysine (Lys), phenylalanine (Phe), threonine (Thr) and valine (Val)—and seven non-essential AAs (AA_{NESS})— alanine (Ala), glycine (Gly), serine (Ser), proline (Pro), aspartic acid (Asp)/asparagine (Asn) referred to here as Asx, glutamic acid (Glu)/glutamine (Gln) referred to here as Glx and tyrosine (Tyr; Figures S1–S2). Gly, Ser and Tyr $\delta^{13}\text{C}$ values were not measured in some samples due to poor peak shape or coelution with other peaks (e.g. Ser coeluted with a large contaminant peak in some plant samples). We also measured the $\delta^{15}\text{N}$ values of these AAs in a subset of

producers ($n = 28$; Figure S3) but could only measure Tyr $\delta^{15}\text{N}$ values in about half of these samples due to poor peak shape.

AA $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values were measured in duplicate on a Thermo Scientific Trace 1310 outfitted with a $60\text{ m} \times 0.32\text{ mm}$ ID BPX5 $\times 1.0\text{ }\mu\text{m}$ column and GC Isolink II coupled to a Delta V Plus isotope ratio mass spectrometer at UNM-CSI. AA $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ measurements were made separately. The in-house AA reference material was analysed every two samples for $\delta^{13}\text{C}$ analysis and bracketed every sample for $\delta^{15}\text{N}$ analysis. For all samples, the SD of AA_{ESS} $\delta^{13}\text{C}$ values and AA $\delta^{15}\text{N}$ values were below 0.8‰ across injections. For the in-house AA reference material, mean within-run SD ranged from 0.2‰ (Ile) to 0.4‰ (Tyr) for $\delta^{13}\text{C}$ and 0.3‰ (Ile) to 0.5‰ (Tyr) for $\delta^{15}\text{N}$ (Table S1). Mean $\delta^{13}\text{C}$ values of each AA were calculated across injections for every sample and corrected to account for the carbon added during derivatization (Equation S2); AA $\delta^{15}\text{N}$ corrections are simpler because no nitrogen is added during derivatization (Equation S3).

2.4 | Statistical analysis

We measured bulk tissue $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values and weight per cent C:N ratios in 57 C_3 plants, 21 C_4 plants, 16 CAM plants and 15 green algae samples. We measured AA $\delta^{13}\text{C}$ values in 60 C_3 plants, 21 C_4 plants, 16 CAM plants and 15 green algae samples, and AA $\delta^{15}\text{N}$ values in nine C_3 plants, 10 C_4 plants, five CAM plants and four green algae samples. The $\delta^{13}\text{C}$ values of AA_{ESS} (Ile, Leu, Lys, Phe, Thr and Val) and three AA_{NESS} (Ala, Asx and Gly) were included in statistical analyses. Two source AAs (Phe and Lys), two trophic AAs (Glu and Pro) and one metabolic AA (Thr) were included in statistical analyses of $\delta^{15}\text{N}$ values to examine variation in β -values. We tested for normality in each variable of interest per producer group using the Shapiro–Wilk test and for homogeneity of variance in each variable of interest across producer groups using Levene’s test (R packages STATS and CAR). We assessed differences among producer groups using Kruskal–Wallis and pairwise Wilcoxon rank-sum tests (R package STATS).

We characterized the AA_{ESS} $\delta^{13}\text{C}$ fingerprints of our producer groups via multivariate techniques. Following previous studies (e.g. Larsen et al., 2013), we used linear discriminant analysis (LDA) on AA_{ESS} $\delta^{13}\text{C}$ data (package MASS). This approach includes a leave-one-out, cross-validation procedure to obtain an error rate measure for our classifier samples, which provides an estimate of how distinct producer groups are in multivariate AA_{ESS} $\delta^{13}\text{C}$ space. We were concerned about meeting the normal distribution and equal variance assumptions of LDA (Zuur et al., 2007), so we also verified our results with flexible discriminant analysis (FDA; R package MDA).

To explore possible biochemical mechanisms for the patterns we observed in our LDA, we used the KEGG PATHWAY Database (Kanehisa et al., 2014) to define the pathways for AA_{ESS} synthesis, using *Populus trichocarpa* and *Solanum lycopersicum* as C_3 plant references, *Zea mays* as a C_4 plant reference and *Chlamydomonas reinhardtii* as a green algae reference. We did not find a suitable CAM

plant reference in KEGG, so we assumed CAM AA_{ESS} synthesis pathways were similar to those of our C_3 and C_4 plant references, which did not differ substantially from each other. We then subtracted AA precursor ($\text{AA}_{\text{precursor}}$) $\delta^{13}\text{C}$ values from AA_{ESS} product ($\text{AA}_{\text{product}}$) $\delta^{13}\text{C}$ values to calculate mean ($\pm\text{SD}$) $\Delta^{13}\text{C}_{\text{productAA-precursorAA}}$ for each producer group. We used Ala $\delta^{13}\text{C}$ values as a proxy for those of pyruvate, a precursor for the synthesis of Val and Leu, as no carbon is added or removed during the amination of pyruvate to form Ala (Scott et al., 2006). There is likely some isotope effect associated with Ala synthesis due to the preferential amination of isotopically lighter pyruvate molecules, which we did not account for. We also used Ala $\delta^{13}\text{C}$ values as a proxy for those of phosphoenolpyruvate (PEP), which is dephosphorylated to pyruvate in the last step of glycolysis (Garrett & Grisham, 2017) and is an important precursor for the synthesis of Phe. We used Kruskal–Wallis and pairwise Wilcoxon rank-sum tests to test for differences in $\Delta^{13}\text{C}_{\text{productAA-precursorAA}}$ among producer groups. Additionally, we used linear regressions and 95% confidence intervals (*lm* and *confit* functions in R package STATS) to determine if the relationships between $\delta^{13}\text{C}_{\text{productAA}}$ and $\delta^{13}\text{C}_{\text{precursorAA}}$ were significant across producer groups. Specifically, we looked at the following AA pairings: Phe–Ala, Val–Ala, Leu–Ala, Thr–Asx, Ile–Thr, Ile–Ala, Lys–Asx and Lys–Ala (for all groups) and Thr–Gly (for plants only).

We calculated AA $\delta^{15}\text{N}$ β -values by subtracting source AA $\delta^{15}\text{N}$ values from trophic and metabolic AA $\delta^{15}\text{N}$ values for the following AA pairings: Glx–Phe, Pro–Phe, Thr–Phe, Glx–Lys, Pro–Lys and Thr–Lys. We tested for differences in β -values among producer groups using Kruskal–Wallis and pairwise Wilcoxon rank-sum tests. We also performed linear regressions to compare bulk tissue $\delta^{15}\text{N}$ values with Phe, Lys, Glx, Pro and Thr $\delta^{15}\text{N}$ values (*lm* function in R package STATS).

3 | RESULTS

3.1 | Bulk tissue $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values

Bulk tissue $\delta^{13}\text{C}$ values (Figure 1; Table S2) differed among all producer groups ($\chi^2 = 81.15$, $p < 0.001$; Table S3). CAM plants had the highest mean ($\pm\text{SD}$) $\delta^{13}\text{C}$ values ($-12.8 \pm 1.1\text{‰}$), followed by C_4 plants ($-14.0 \pm 0.5\text{‰}$), green algae ($-24.6 \pm 2.1\text{‰}$) and C_3 plants ($-27.3 \pm 2.1\text{‰}$). Green algae had significantly higher bulk tissue $\delta^{15}\text{N}$ values than terrestrial plants ($\chi^2 = 16.69$, $p < 0.001$; Table S3). C:N ratios of CAM plants (46.9 ± 20.1) were significantly higher than those of all other groups, while those of green algae (9.6 ± 2.8) were lower ($\chi^2 = 51.83$, $p < 0.001$). C:N ratios of C_3 (27.8 ± 14.1) and C_4 (22.8 ± 7.2) plants did not statistically differ ($p = 0.38$; Table S3).

3.2 | Essential amino acid $\delta^{13}\text{C}$ values

There were strong statistical differences in AA_{ESS} $\delta^{13}\text{C}$ values among producer groups (Figure 2; Tables S4 and S5). Leu and Phe $\delta^{13}\text{C}$

FIGURE 1 Bulk tissue $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values of terrestrial and freshwater producers from the northern Chihuahuan Desert (New Mexico, USA). Data for C_3 plants (leaves; green circles), C_4 plants (seeds or leaves; yellow circles), CAM plants (paddles; black circles) and filamentous green algae (blue triangles). Translucent coloured boxes indicate the $\delta^{13}\text{C}$ ranges for C_3 plants (green), filamentous green algae (blue), C_4 plants (yellow) and CAM plants (black)

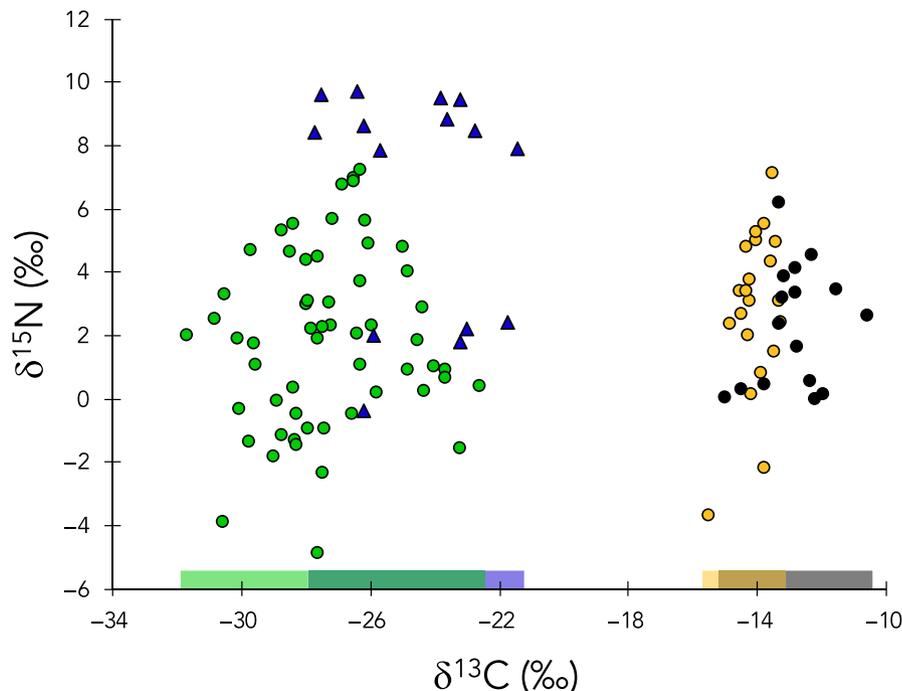
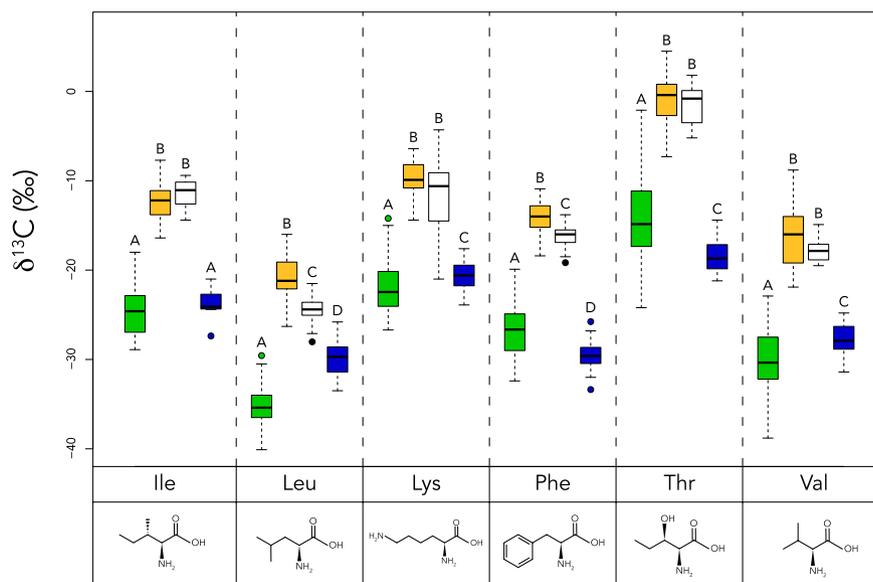


FIGURE 2 Box plots of $\text{AA}_{\text{ESS}} \delta^{13}\text{C}$ values of terrestrial and freshwater producers from the northern Chihuahuan Desert (New Mexico, USA). $\delta^{13}\text{C}$ values of six AA_{ESS} —isoleucine (Ile), leucine (Leu), lysine (Lys), phenylalanine (Phe), threonine (Thr) and valine (Val)—for C_3 plants (green), C_4 plants (yellow), CAM plants (black outline) and filamentous green algae (blue). Black bars represent medians and circles represent outliers. Letters (A, B, C, D) denote statistically significant differences among producer groups (Table S5)



values differed among all producer group pairings (Leu: $\chi^2 = 88.90$, $p < 0.001$; Phe: $\chi^2 = 80.55$, $p < 0.001$), while Ile $\delta^{13}\text{C}$ values differed among all pairings except C_3 plants–algae and CAM plants– C_4 plants ($\chi^2 = 74.79$, $p < 0.001$). Lys, Thr and Val $\delta^{13}\text{C}$ values differed among all pairings except CAM plants– C_4 plants (Lys: $\chi^2 = 71.61$, $p < 0.001$; Thr: $\chi^2 = 76.38$, $p < 0.001$; Val: $\chi^2 = 77.40$, $p < 0.001$). Only Leu $\delta^{13}\text{C}$ values in C_3 plants were significantly different between the Bosque and Sevilleta LTER sites (Table S6).

3.3 | Essential amino acid $\delta^{13}\text{C}$ fingerprints

Green algae Ile and C_3 plant Lys $\delta^{13}\text{C}$ values exhibited non-normal distributions, while Ile, Lys, Phe, Thr and Val $\delta^{13}\text{C}$ values displayed

unequal variance among producer groups (Table S5). These findings violate assumptions of LDA, so we verified our results with FDA. FDA results did not differ substantially from LDA results (Figure S4; Tables S7 and S8), so we focused on LDA for consistency with previous studies (Elliott Smith et al., 2018; Fox et al., 2019; Larsen et al., 2013). LDA on $\text{AA}_{\text{ESS}} \delta^{13}\text{C}$ values yielded an overall 99% successful reclassification rate among all producer groups (Figure 3). All green algae (15/15), C_3 plant (60/60) and CAM plant (16/16) samples correctly reclassified, while only 1 of 21 C_4 plants misclassified (as a CAM plant; Table S9). The first linear discriminant (LD1) explained 68.2% of the variation in our dataset and was driven predominantly by Leu $\delta^{13}\text{C}$ values (Table S10). LD2 and LD3 explained a further 27.6% and 4.2% of the variance, respectively, with Leu and Phe (LD2) and Ile (LD3) $\delta^{13}\text{C}$ values being the most important variables (Table

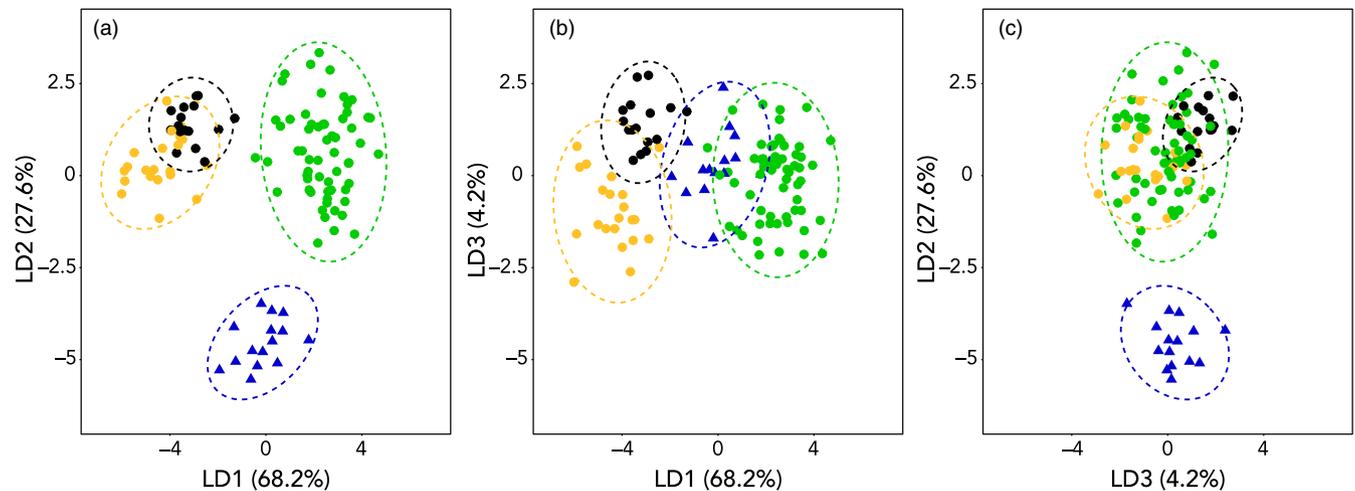


FIGURE 3 AA_{ESS} $\delta^{13}\text{C}$ fingerprints of terrestrial and freshwater producers from the northern Chihuahuan Desert (New Mexico, USA). Linear discriminant analysis plots generated using $\delta^{13}\text{C}$ values of six AA_{ESS} (isoleucine, leucine, lysine, phenylalanine, threonine and valine) for C₃ plants (green circles), C₄ plants (yellow circles), CAM plants (black circles) and filamentous green algae (blue triangles). (A) Linear discriminant 1 (LD1) vs. LD2. (B) LD1 vs. LD3. (C) LD3 vs. LD2. Dotted lines represent 95% confidence intervals. LD1 explains 68.2% of the variation, LD2 explains 27.6% and LD3 explains 4.2%. The overall successful reclassification rate is 99% (Table S9)

S10). We also performed LDA at the taxon level for taxa with sample sizes >7 to determine the lowest taxonomic resolution of this technique and obtained an overall successful reclassification rate of 64% (Figure S5; Tables S11 and S12).

3.4 | $\Delta^{13}\text{C}_{\text{productAA-precursorAA}}$ calculations

Ala $\delta^{13}\text{C}$ values differed among all producer groups except C₄ and CAM plants ($\chi^2 = 77.96$, $p < 0.001$; Figure S6; Tables S13 and S14). Gly and Asx $\delta^{13}\text{C}$ values differed among all producer groups except C₃ plants and green algae (Gly: $\chi^2 = 46.46$, $p < 0.001$; Asx: $\chi^2 = 60.41$, $p < 0.001$; Figure S3; Tables S13 and S14). Linear regressions of AA_{product} and AA_{precursor} $\delta^{13}\text{C}$ values were significant for all combinations except Val-Ala, Thr-Gly and Lys-Asx in CAM plants and Thr-Asx in green algae (Table S15). C₃ and C₄ plant $\Delta^{13}\text{C}_{\text{productAA-precursorAA}}$ for all AA combinations were statistically indistinguishable (Figure 4; Figure S7; Table S16). $\Delta^{13}\text{C}_{\text{Lys-Asx}}$ did not differ among any producers (Figure 4; Table S16).

3.5 | Source and trophic amino acid $\delta^{15}\text{N}$ values

Despite having the highest bulk tissue $\delta^{15}\text{N}$ values, green algae had the lowest Phe $\delta^{15}\text{N}$ values, which differed from those of C₃ and C₄ plants ($\chi^2 = 10.60$, $p = 0.014$; Figure S8; Tables S17 and S18). Lys, Glx and Pro $\delta^{15}\text{N}$ values were not statistically different among producer groups (Lys: $\chi^2 = 10.06$, $p = 0.018$; Glx: $\chi^2 = 5.98$, $p = 0.11$; Pro: $\chi^2 = 4.98$, $p = 0.17$; Figure S8; Tables S17 and S18). Thr $\delta^{15}\text{N}$ values were distinct only for green algae ($\chi^2 = 12.76$, $p = 0.005$; Figure S8; Tables S17 and S18). $\beta_{\text{Glx-Phe}}$, $\beta_{\text{Pro-Phe}}$, $\beta_{\text{Thr-Phe}}$ and $\beta_{\text{Thr-Lys}}$ values were significantly higher for green algae than for C₃, C₄ and CAM plants, which were not statistically different among each

other ($\beta_{\text{Glx-Phe}}$: $\chi^2 = 10.32$, $p = 0.016$; $\beta_{\text{Pro-Phe}}$: $\chi^2 = 12.29$, $p = 0.006$; $\beta_{\text{Thr-Phe}}$: $\chi^2 = 10.94$, $p = 0.012$; $\beta_{\text{Thr-Lys}}$: $\chi^2 = 11.81$, $p = 0.008$; Figure 5; Tables S19 and S20). $\beta_{\text{Glx-Lys}}$ and $\beta_{\text{Pro-Lys}}$ values did not differ among producer groups ($\beta_{\text{Glx-Lys}}$: $\chi^2 = 3.22$, $p = 0.36$; $\beta_{\text{Pro-Lys}}$: $\chi^2 = 7.26$, $p = 0.064$; Figure 5; Tables S19 and S20).

4 | DISCUSSION

4.1 | Distinguishing among producers using essential amino acid $\delta^{13}\text{C}$ fingerprints

We found a near-perfect separation of our producer groups using LDA with a successful reclassification rate of 99% (Figure 3; Table S9). This is an improvement over bulk tissue isotope analysis, where our producer groups had overlapping $\delta^{13}\text{C}$ values (Figure 1). Below, we discuss biochemical mechanisms that likely contribute to these distinct AA_{ESS} $\delta^{13}\text{C}$ fingerprints, a topic which has received little attention in previous studies analysing freshwater and terrestrial primary producers (Bowes et al., 2020; Liew et al., 2019; Thorp & Bowes, 2017).

The greater the complexity of a biological molecule, the more opportunity there is for kinetic isotope effects associated with enzymatic reactions during de novo synthesis, leading to an increased likelihood for divergence in $\delta^{13}\text{C}$ values among producers with different photosynthetic and biochemical pathways. Several processes contribute to divergence in AA_{ESS} $\delta^{13}\text{C}$ values among producers. First, the $\delta^{13}\text{C}$ values of precursors strongly influence the $\delta^{13}\text{C}$ values of AA_{ESS} products. For example, relatively simple precursors (e.g. pyruvate) used to synthesize the more complex carbon skeletons of AA_{ESS} have lower $\delta^{13}\text{C}$ values in C₃ plants than in C₄ and CAM plants (Figure S6; Tables S13 and S14). Second, the supply and demand of AAs needed for metabolism influence their pool sizes and

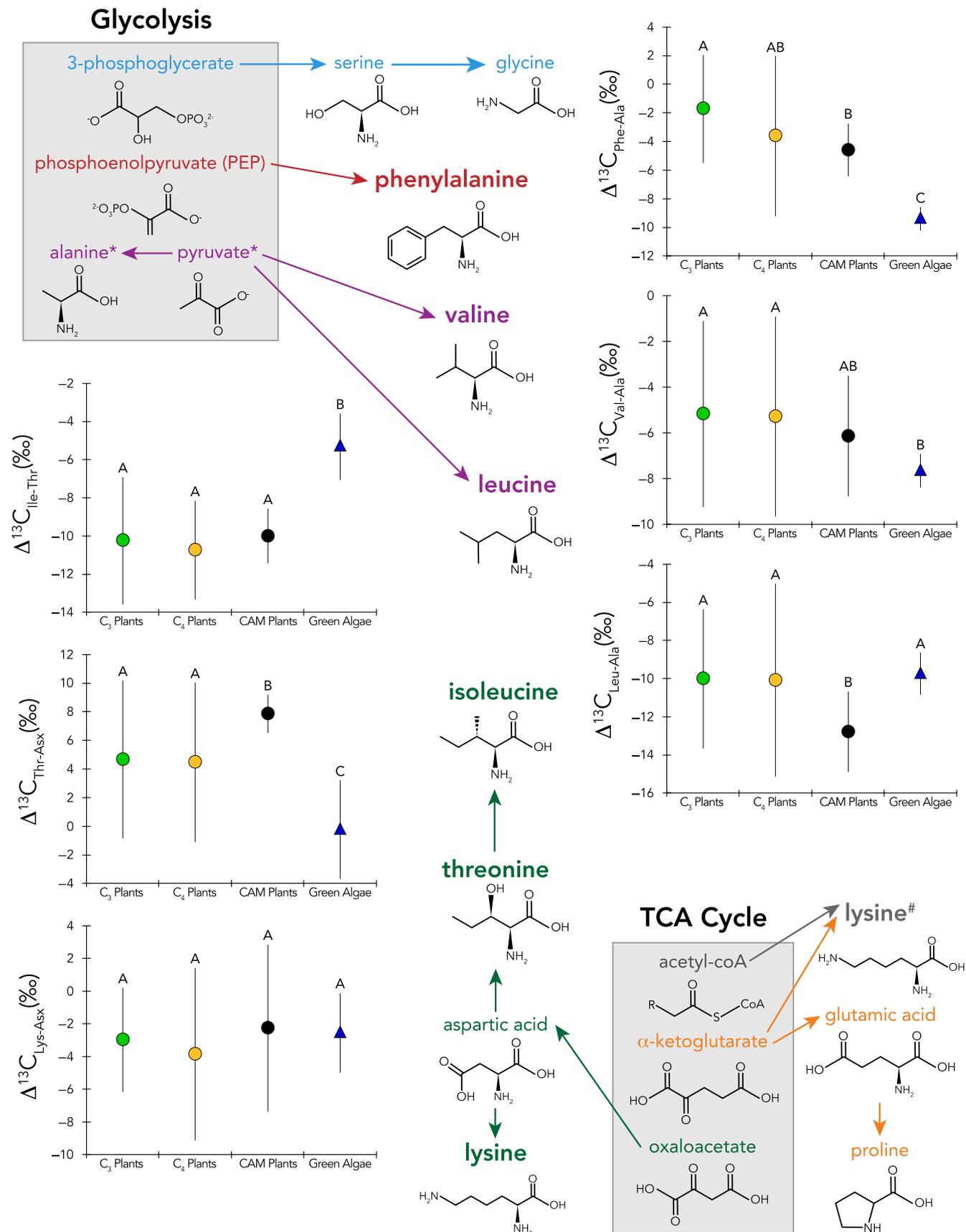


FIGURE 4 Isotopic relationships between AA_{ESS} and their molecular precursors. Precursors for AA synthesis originate from glycolysis and the tricarboxylic acid (TCA) cycle. AA_{ESS} are in bold. Asterisks (*) indicate Ala δ¹³C values are a proxy for those of pyruvate. The hash (#) following lysine synthesized from acetyl-coA indicates this synthesis pathway only occurs in fungi and protists. Inset graphs display the means (circles and triangles) and standard deviations (black lines) of the differences in the δ¹³C values of product AA_{ESS} and precursor AA, which are proxies for the isotopic discrimination associated with specific synthesis pathways (Δ¹³C_{productAA-precursorAA}). Letters (A, B, C) denote statistically significant differences among groups (Table S16)

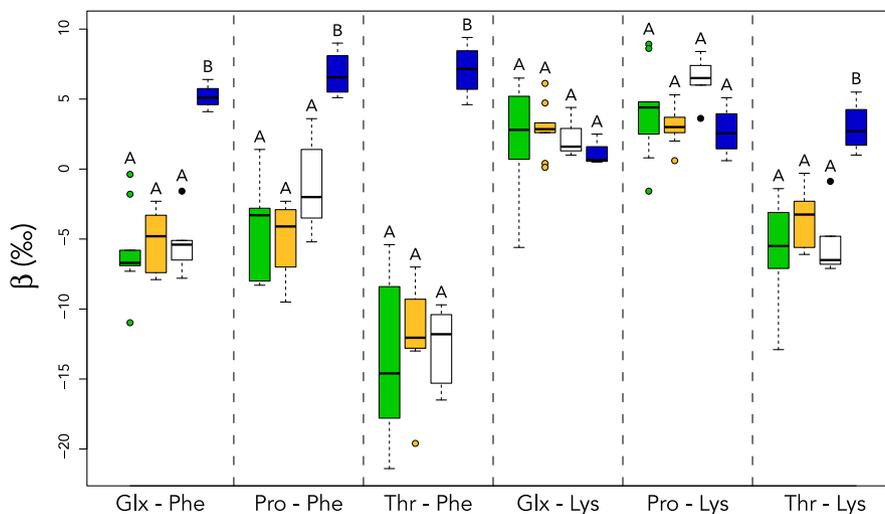


FIGURE 5 β -Values for terrestrial and freshwater producers from the northern Chihuahuan Desert (New Mexico, USA). Box plots of β -values for C_3 plants (green), C_4 plants (yellow), CAM plants (black outline) and filamentous green algae (blue). Black bars represent medians and circles represent outliers. Letters (A, B) denote statistically significant differences among groups (Table S20)

turnover rates, which can vary among producer groups (Hildebrandt et al., 2015). These factors contribute to variation in AA_{ESS} $\delta^{13}C$ values due to two key phenomena: (a) larger precursor pool sizes create more opportunity for isotopic discrimination during the de novo synthesis of AA_{ESS} than smaller precursor pool sizes and (b) faster turnover rates lead to less isotopic discrimination (Fry, 2006). Third, isotopically lighter (^{13}C -depleted) precursors are preferentially selected by enzymes to varying extents (Takizawa et al., 2020). If the specific enzymes used in AA_{ESS} synthesis pathways differ among producer groups, this could impact the resulting net isotopic discrimination, as different enzymes have unique kinetic-based fractionation factors (Hayes, 2001). Lastly, AAs and the molecules from which they are synthesized also serve as precursors for other secondary metabolites (Hildebrandt et al., 2015). Catabolism of AA_{ESS} for secondary metabolite synthesis involves kinetic isotope effects, where AA_{ESS} containing light isotopes (e.g. ^{12}C or ^{14}N) are preferentially catabolized, leaving the remaining pool of AA_{ESS} isotopically heavier.

There are five precursor families for AA synthesis (**AA_{ESS}** in bold; Garrett & Grisham, 2017): (1) pyruvate (Ala, **Val** and **Leu**), (2) oxaloacetate (Asx, **Thr**, **Ile** and **Lys** [for plants, bacteria and algae]), (3) PEP and erythrose-4-P (Tyr and **Phe**), (4) α -ketoglutarate (Glx, Pro and **Lys** [for some fungi and protists]) and (5) 3-phosphoglycerate (Ser and Gly; Figures 4, 6, and S7). Ala $\delta^{13}C$ values, a proxy for pyruvate and PEP $\delta^{13}C$ values, were statistically different among all producer group pairings except C_4 and CAM plants (Tables S13 and S14). Thus, any differences in the $\delta^{13}C$ values of AA_{ESS} synthesized from pyruvate or PEP between C_4 and CAM plants must be due to the differences in the $\delta^{13}C$ values of other precursors (e.g. acetyl coA) that add carbon during de novo synthesis, vastly different AA supply and demand rates, or the use of unique enzymes.

In general, C_3 and C_4 plants did not have statistically different $\Delta^{13}C_{productAA-precursorAA}$ for any AA pairings, while CAM plant and algae $\Delta^{13}C_{productAA-precursorAA}$ varied. Similar precursor $\delta^{13}C$ values

but distinct $\Delta^{13}C_{productAA-precursorAA}$ among producer groups indicate downstream biochemical processes drive the variation in product AA_{ESS} $\delta^{13}C$ values. These findings also stress the importance of the relative differences in $\delta^{13}C$ values among AA_{ESS} in creating unique isotopic fingerprints. In particular, divergences in the $\delta^{13}C$ values of Leu, Phe and Ile drive the unique AA_{ESS} patterns observed among producer groups (Figure 3).

Two AA_{ESS} —Val and Leu—are synthesized from pyruvate via four or five and seven to nine enzymatic reactions, respectively (Garrett & Grisham, 2017; Kanehisa et al., 2014). Val contains five carbons, three of which come from pyruvate. The other two originate from the hydroxyethyl group of hydroxyethyl-thiamine pyrophosphate, a coenzyme which itself is synthesized from pyruvate and thiamine pyrophosphate in one enzymatic reaction (Figure 6; Garrett & Grisham, 2017; Kanehisa et al., 2014). C_3 , C_4 and CAM plants had statistically indistinguishable $\Delta^{13}C_{Val-Ala}$, while green algae had lower $\Delta^{13}C_{Val-Ala}$ than C_3 and C_4 plants (Figure 4; Table S16). Because C_3 plants had the lowest Ala $\delta^{13}C$ values, they likely also had the lowest pyruvate $\delta^{13}C$ values, indicating there may be greater kinetic isotope effects associated with Val synthesis in green algae than in C_3 plants. The first three or four reactions of Leu and Val synthesis are identical, after which two more carbons are added from acetyl-CoA, while one that ultimately originated from the carboxyl group of a pyruvate is lost as CO_2 , to complete Leu synthesis (Figure 6; Garrett & Grisham, 2017; Kanehisa et al., 2014). Thus, four of Leu's six carbons come from pyruvate and two from acetyl-CoA. Our data suggest that the additional two reactions that add and remove carbon during Leu synthesis led to increased isotopic discrimination, such that $\Delta^{13}C_{Leu-Ala}$ were $\sim 7\%$ lower in CAM plants, $\sim 5\%$ lower in C_3 and C_4 plants, and $\sim 2\%$ lower in green algae in comparison to $\Delta^{13}C_{Val-Ala}$ for these producer groups (Figure 4). CAM plants had lower $\Delta^{13}C_{Leu-Ala}$ than C_3 plants, C_4 plants and green algae, which were statistically indistinguishable (Figure 4; Table S16). The variation observed in Ala $\delta^{13}C$ values and $\Delta^{13}C_{Leu-Ala}$ highlights why Leu $\delta^{13}C$ values differed

among all producer groups and drove LD1, which explained 68.2% of the variation among producer groups in the LDA.

The oxaloacetate family includes three (Thr, Ile and Lys) of the six AA_{ESS} routinely used to define fingerprints (Larsen et al., 2013). Oxaloacetate is first transaminated with Glu to form Asp (Scott et al., 2006), from which Thr, Ile and Lys are synthesized (Figure 6). Asx $\delta^{13}\text{C}$ values differed among all producer groups except C₃ plants and green algae (Figure S6; Tables S13 and S14). Thus, differences in the $\delta^{13}\text{C}$ values of Thr, Ile and Lys between C₃ plants and green algae must be explained by the $\delta^{13}\text{C}$ values of other precursors that contribute carbon, different enzymatic kinetic isotope effects, or varied AA supply and demand rates. Thr is synthesized from Asp via a pathway of five enzymatic reactions, none of which add or remove carbon (Garrett & Grisham, 2017; Kanehisa et al., 2014). It is important to note that homoserine is an intermediary in the synthesis of both Thr and Met (Kanehisa et al., 2014; Garrett & Grisham, 2017), another AA_{ESS} less frequently analysed in CSIA-based studies. Isotopic discrimination associated with Thr synthesis ($\Delta^{13}\text{C}_{\text{Thr-Asx}}$) is then regulated by enzymes and/or the pool sizes and turnover rates of Asp and Thr. As such, the variation in $\Delta^{13}\text{C}_{\text{Thr-Asx}}$ is surprising; CAM plants had higher $\Delta^{13}\text{C}_{\text{Thr-Asx}}$ than C₃ and C₄ plants, while green algae had lower $\Delta^{13}\text{C}_{\text{Thr-Asx}}$ (Figure 4). Interestingly, Thr $\delta^{13}\text{C}$ values were higher than Asx $\delta^{13}\text{C}$ values in plants, potentially indicating extensive catabolism of Thr for Ile synthesis or to form Gly and acetaldehyde, a reversible reaction and thus, an alternative Thr synthesis pathway (Hildebrandt et al., 2015) that appears to be used by C₃ and C₄ plants (Figure S7; Table S15). However, CAM plants displayed no correlation between Thr and Gly $\delta^{13}\text{C}$ values (Table S15), suggesting the positive $\Delta^{13}\text{C}_{\text{Thr-Asx}}$ value for this group is caused by extensive catabolism of Thr for Ile synthesis. Ile is synthesized from Thr via a pathway involving five or six enzymatic reactions. The first converts Thr into α -ketobutyrate, which then receives two carbon atoms from the hydroxyethyl group of the coenzyme hydroxyethyl-thiamine pyrophosphate (Figure 6; Garrett & Grisham, 2017). Algae had significantly higher $\Delta^{13}\text{C}_{\text{Ile-Thr}}$ than terrestrial plants, which were statistically indistinguishable (Figure 4; Table S16). To examine the influence of adding two carbon atoms from pyruvate on Ile $\delta^{13}\text{C}$ values, we also compared Ile and Ala $\delta^{13}\text{C}$ values; green algae exhibited slightly lower $\Delta^{13}\text{C}_{\text{Ile-Ala}}$ than terrestrial plants (Figure S7; Table S16). The differences in the $\delta^{13}\text{C}$ values of the two precursors—Thr and pyruvate—and the discrimination factors ($\Delta^{13}\text{C}_{\text{Ile-Thr}}$ and $\Delta^{13}\text{C}_{\text{Ile-Ala}}$) associated with Ile synthesis help explain the divergence of Ile $\delta^{13}\text{C}$ values among producer groups and why they drove variation along LD3 (Figure 3).

The last AA_{ESS} in the oxaloacetate family, Lys, is synthesized from Asp via seven to 10 enzymatic reactions (Kanehisa et al., 2014; Garrett & Grisham, 2017). In this pathway, three carbons are added from pyruvate and one is lost as CO₂ from either the carboxyl group of pyruvate or the α -carboxyl of Asp—both losses occur in equal proportions due to the symmetric nature of the intermediary (Figure 6; Garrett & Grisham, 2017). All producer groups had the same $\Delta^{13}\text{C}_{\text{Lys-Asx}}$ (Figure 4; Table S16). The low variability in $\Delta^{13}\text{C}_{\text{Lys-Asx}}$

among producer groups is consistent with the low degree of influence Lys $\delta^{13}\text{C}$ values had on the linear discriminants in LDA.

Phenylalanine has two precursors: PEP, an intermediary in glycolysis, and erythrose-4-P, a compound produced in the Calvin cycle and pentose phosphate pathway. PEP (three carbons) and erythrose-4-P (four carbons) first undergo a reaction to form 2-keto-3-deoxyarabino-heptulosonate-7-P, which is then converted to Phe through an additional nine enzymatic reactions (Figure 6; Kanehisa et al., 2014; Garrett & Grisham, 2017). Another three carbon atoms are added from PEP during its reaction with shikimate-5-P (Figure 6; Kanehisa et al., 2014; Garrett & Grisham, 2017). Prephenate is produced in the eighth reaction, after which there are two alternative pathways: one that produces phenylpyruvate as an intermediate and one that produces 4-hydroxyphenylpyruvate or arogenate, which are also immediate precursors to Tyr (Kanehisa et al., 2014). In both pathways, a carbon atom is lost as CO₂ (Figure 6). $\Delta^{13}\text{C}_{\text{Phe-Ala}}$, which is a proxy for $\Delta^{13}\text{C}_{\text{Phe-PEP}}$, as Ala $\delta^{13}\text{C}$ values are a proxy for those of PEP, was lowest for green algae (Figure 4), likely the result of several factors. First, the $\delta^{13}\text{C}$ composition of the precursors erythrose-4-P and PEP are likely very different among producer groups, with C₃ plants and green algae having the lowest $\delta^{13}\text{C}$ values. Second, there are likely isotope effects associated with different supply and demand rates for the precursor PEP in C₃ plants and green algae versus C₄ plants and CAM plants, which use it for initial CO₂ fixation. Only erythrose-4-P is involved in photosynthesis in C₃ plants and green algae, whereas both erythrose-4-P and PEP are involved in C₄ and CAM photosynthesis (Lambers et al., 2008). Finally, Phe is a precursor to many secondary metabolites (e.g. phenolics; Lambers et al., 2008). Phe catabolism for secondary metabolite synthesis occurs to varying extents in different producer groups and likely increases the $\delta^{13}\text{C}$ of residual Phe, although the effects of phenolic production on isotopic discrimination have only been documented for $\delta^{15}\text{N}$ to date (Kendall et al., 2019). The complicated balance between Phe biosynthesis and catabolism for structural and secondary compound synthesis likely impacts its $\delta^{13}\text{C}$ values, as evidenced by the variation observed in $\Delta^{13}\text{C}_{\text{Phe-Ala}}$ (Figure 4), and provides a possible explanation for the importance of Phe $\delta^{13}\text{C}$ values in driving LD2 in our analysis.

4.2 | Evaluating β -values and the potential of AA $\delta^{15}\text{N}$ analysis

We found a wide range (~12‰) in bulk tissue $\delta^{15}\text{N}$ values within and among producer groups (Figure 1) that is likely driven by a combination of factors, including variation in rooting depth, use of different forms of inorganic nitrogen (N₂, NH₄⁺ and NO₃⁻) and soil moisture content (Austin & Vitousek, 1998; Evans, 2001). This variability highlights why the analysis of [baseline] primary producers is crucial when using bulk tissue $\delta^{15}\text{N}$ values to study consumer diet composition and TL. TL estimates based on AA $\delta^{15}\text{N}$ analysis may negate the need for baseline sampling (Chikaraishi et al., 2009); however, this approach is dependent on accurate estimates of β -values

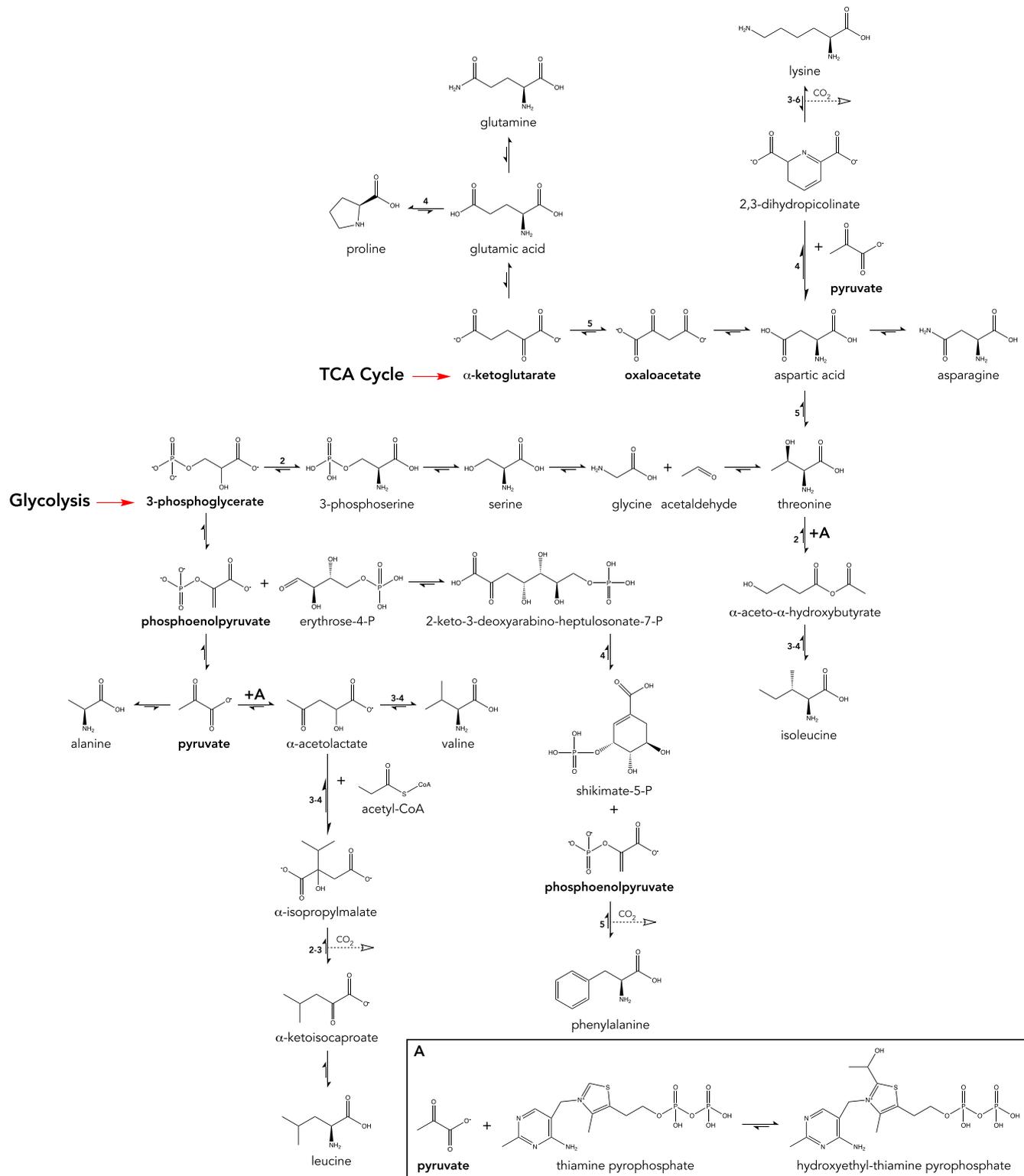


FIGURE 6 Simplified amino acid biosynthetic pathways. Only reactions in which carbon and nitrogen are added or lost are shown. Numbers next to reaction arrows indicate the number of enzymatic reactions between the molecules displayed; a single enzymatic reaction is represented if no number is present. Panel A depicts the synthesis of the coenzyme hydroxyethyl-thiamine pyrophosphate. Figure made using reactions from the KEGG PATHWAY Database (Kanehisa et al., 2014) and Garrett and Grisham (2017)

for appropriate trophic-source AA pairs and a recent meta-analysis found β -values are considerably more variable than previously thought (Ramirez et al., 2021).

We found considerable variation in source AA $\delta^{15}\text{N}$ values within and among taxa, especially for the canonical source AA Phe, which had a 22.6% range across taxa (Figure S8; Table S17). Additionally,

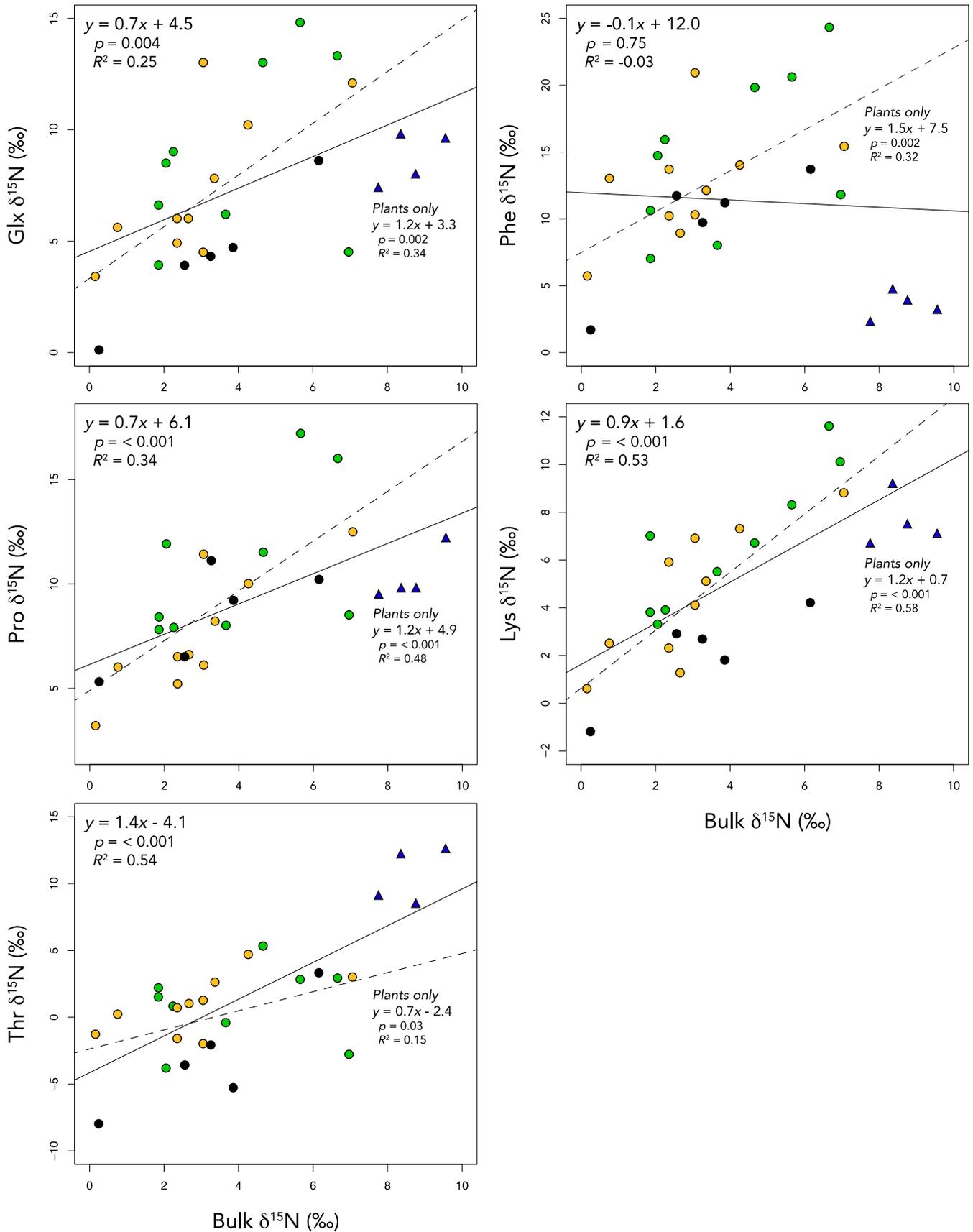


FIGURE 7 Bulk tissue $\delta^{15}\text{N}$ and individual AA $\delta^{15}\text{N}$ linear regressions for terrestrial and freshwater producers from the northern Chihuahuan Desert (New Mexico, USA). The equation, $y = mx + b$, where m = slope and b = y -intercept, p -value and R^2 of the regression are located in the upper left corner of each plot. Linear regressions were also made for terrestrial plants only (dashed line)

while linear regressions of producer bulk tissue and Glx, Pro, Thr and Lys $\delta^{15}\text{N}$ values were all statistically significant, the relationship between bulk tissue and Phe $\delta^{15}\text{N}$ values was not (Figure 7). Phe is a precursor to many secondary metabolites (e.g. tannins) and structural compounds (e.g. lignin) that are critical for plants and algae (Aigner et al., 2013; Lambers et al., 2008; Popper et al., 2011). The extent of Phe catabolism for the synthesis of secondary compounds, which results in ^{15}N enrichment in residual Phe (Kendall et al., 2019), varies widely depending on a variety of factors, including abiotic stress (Sharma et al., 2019), but likely occurs to a greater extent in terrestrial plants that need to produce more structural compounds than in aquatic algae. In our study, C_3 plants exhibited the largest range in Phe $\delta^{15}\text{N}$ values (17.3‰). *Krascheninnikovia lanata*, a C_3 species belonging to Amaranthaceae, a family known for its synthesis of diverse phenolic acids and flavonoids (Mroczek, 2015), displayed the highest Phe $\delta^{15}\text{N}$ values (Figure S8; Table S17). Another source AA, Lys, is also a precursor to molecules involved in plant immunity and increases in concentration during abiotic stress to act as an alternative respiratory substrate (Galili et al., 2001; Zeier, 2013). However, we found Lys $\delta^{15}\text{N}$ values were less variable (range = 12.9‰; Figure S8; Table S17) within and among producer groups in comparison to Phe, potentially indicating Lys is catabolized less than Phe in the suite of producers we analysed. There was also a strong linear relationship between bulk tissue and Lys $\delta^{15}\text{N}$ values (Figure 7), further suggesting the demand on the Lys pool for the synthesis of secondary compounds is likely not as substantial as it is for the Phe pool. It is worth noting that when excluding green algae, plant bulk tissue and Phe $\delta^{15}\text{N}$ values were significantly correlated ($p = 0.002$, $R^2 = 0.32$), although plant bulk tissue and Lys $\delta^{15}\text{N}$ values were still more strongly correlated ($p < 0.001$, $R^2 = 0.58$; Figure 7). These results indicate Lys may be a better source AA for calculating producer β -values and consumer TL in terrestrial and freshwater ecosystems; Lys $\delta^{15}\text{N}$ values may be especially useful for food webs that rely on a mixture of terrestrial and aquatic resources. Previous studies have also identified methionine (Met) as a promising source AA for mixed terrestrial–marine systems because $\beta_{\text{Glx-Met}}$ values did not differ between a small set of cultivated terrestrial and wild marine producers (Ishikawa et al., 2018).

While previous work on a small number of cultivated and fertilized plants, which typically receive substantially more nitrogen than is available to wild plants, suggested that C_3 and C_4 plants have $\beta_{\text{Glx-Phe}}$ that differ by ~9‰ (Chikaraishi et al., 2010), our dataset of wild-collected producers shows no significant differences in β -values among terrestrial plant types for any of the trophic-source or metabolic-source AA pairings (Figure 5; Tables S19 and S20). This finding simplifies AA $\delta^{15}\text{N}$ -based estimates of consumer TL in many terrestrial ecosystems and makes physiological sense, as the way plants acquire and transport nitrogen does not correlate with their photosynthetic pathway (Werner & Schmidt, 2002).

Our results suggest that $\beta_{\text{Glx-Lys}}$ and $\beta_{\text{Pro-Lys}}$ are the best for estimating the TL of consumers that rely on a combination of terrestrial and aquatic resources, as these β -values do not differ among plants and green algae (Figure 5; Tables S19 and S20). This finding agrees

with the results and recommendations brought forth by Ramirez et al. (2021), who found that β -values calculated using Lys as the source AA were less variable than those calculated using Phe. Glx is central to nitrogen metabolism and is subjected to more transamination reactions than any other AA (O'Connell, 2017). Glx and Pro $\delta^{15}\text{N}$ values are similar among all producer groups, likely because Pro can be converted to Glu in two oxidation steps that do not involve transamination (Hildebrandt et al., 2015). As mentioned above, the role of Phe in the synthesis of secondary metabolites and structural compounds may contribute to differences in the $\delta^{15}\text{N}$ values of this source AA between C_3/C_4 plants and green algae (Table S18), yielding strikingly different β -values between terrestrial plants and freshwater algae when Phe is used as the source AA (Figure 5). Lys $\delta^{15}\text{N}$ values are more analytically challenging to measure than those of Phe due to potential coelution with histidine and Tyr, which is likely why many studies do not report Lys $\delta^{15}\text{N}$ data; but see Figures S1–S3 for example $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ chromatograms from this study with good separation between Tyr and Lys. Given the invariant nature of Lys $\delta^{15}\text{N}$ values relative to those of Phe (Figure S8), we urge researchers to adopt or design analytical protocols that produce Lys $\delta^{15}\text{N}$ data.

4.3 | Moving forward

Although part of the draw of using AA isotope analyses in food web studies is the potential to forgo collection and analysis of primary producers due to the universality of producer $\text{AA}_{\text{ESS}} \delta^{13}\text{C}$ fingerprints and β -values, there are many fundamental questions regarding these important AA isotopic parameters that remain. Perhaps most importantly, how conserved are $\text{AA}_{\text{ESS}} \delta^{13}\text{C}$ fingerprints and β -values across taxa, space and time?

We suggest researchers include the collection and analysis of primary producers when designing food web studies utilizing AA isotope analysis. Below, we provide six main suggestions for future studies that want to incorporate these techniques. (a) Study design should consider analytical (e.g. derivatization and purification) and statistical (e.g. discriminant analysis) methods. For example, the use of LDA to define $\text{AA}_{\text{ESS}} \delta^{13}\text{C}$ fingerprints requires $n + 1$ samples per group for n number of variables (e.g. $\text{AA}_{\text{ESS}} \delta^{13}\text{C}$ values). The minimum number of samples needed to define β -values is not as straightforward; however, statistical inferences would be enhanced with adequate sample sizes ($n = 10$) rather than the limited number of samples ($n = 2\text{--}3$) reported in most studies to date. (b) Studies should aim to adequately sample the taxonomic diversity of primary producers across a habitat or landscape—this includes both the dominant producers in terms of biomass or quantity (e.g. riparian vegetation) and the ecologically important producers that may not be as abundant (e.g. aquatic algae). (c) Researchers should consider using a function-based (e.g. C_3 vs. C_4) strategy when collecting primary producers in ecosystem(s) of interest, as $\text{AA}_{\text{ESS}} \delta^{13}\text{C}$ fingerprints and/or $\delta^{15}\text{N}$ β -values may separate statistically among functional groups, although more work at lower taxonomic levels (e.g. family or genera) is needed to further define the resolution of these tools. (d) The

biochemical and physiological mechanisms underlying variation in AA $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ patterns among primary producers deserve further investigation in carefully controlled laboratory or greenhouse experiments that can manipulate growth conditions (e.g. nutrient availability) and/or measure related variables (e.g. growth rates, AA concentrations and secondary phenolic concentrations). (e) Researchers should aim to measure isotope values from the full suite of AAs their analytical techniques allow, which will further refine the capabilities and limitations of AA-based isotopic approaches in ecological studies. For example, the potential of Lys and Met as useful $\delta^{15}\text{N}$ source AAs, as well as the varied importance of each AA_{ESS} in driving AA_{ESS} $\delta^{13}\text{C}$ fingerprints across studies focused on different functional groups of primary producers (e.g. Lys, Phe, Leu and Val were most important in Larsen et al., 2013; Ile, Lys and Val were most important in Elliott Smith et al., 2021), appeals for future meta-analyses of the full suite of AA that can be reliably measured via GC-C- or LC-IRMS. (f) Finally, to ensure the highest level of data quality, we suggest researchers carefully examine every AA peak in all of their chromatograms, even if they are directly exporting data from Isodat or another IRMS software, to check for coelution and poorly defined peaks or backgrounds. We have found that entering all AA data by hand, peak by peak, allows us to maintain a consistently high level of data quality and identify instrument issues promptly as they arise. We hope the data and recommendations reported here encourage ecologists to think about the structure and function of food webs in terms of essential biomolecules, a crucial currency for higher TL consumers (Ruess & Müller-Navarra, 2019).

5 | CONCLUSIONS

Our results highlight the enhanced discriminatory power of AA_{ESS} $\delta^{13}\text{C}$ analysis for tracing terrestrial and freshwater primary production through food webs and provide new β -value data crucial for estimating the TL of consumers that rely on a combination of these basal resources. Our study is the first to characterize AA_{ESS} $\delta^{13}\text{C}$ fingerprints of wild-collected terrestrial plants and freshwater green algae with large sample sizes (>10) at the landscape scale, and to present strong, but previously unrecognized, differences in fingerprints among C₃, C₄ and CAM plants. Unlike previous work, we found no significant differences in $\beta_{\text{Glx-Phe}}$ among terrestrial plant types. Furthermore, we found $\beta_{\text{Glx-Lys}}$ and $\beta_{\text{Pro-Lys}}$ are indistinguishable among green algae and terrestrial plants, indicating these trophic-source pairings are ideal for estimating TL of consumers relying on a mixture of terrestrial and freshwater resources.

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CONFLICT OF INTEREST

All the authors declare no conflict of interest.

AUTHORS' CONTRIBUTIONS

A.C.B., E.A.E.S. and S.D.N. designed the study; A.C.B. collected and analysed the data; A.C.B., E.A.E.S. and S.D.N. interpreted the data; A.C.B. wrote the manuscript, while E.A.E.S. and S.D.N. edited the manuscript and gave final approval for publication.

PEER REVIEW

The peer review history for this article is available at <https://publons.com/publon/10.1111/1365-2745.13853>.

DATA AVAILABILITY STATEMENT

The bulk tissue and amino acid isotope data presented in this manuscript are archived in the Dryad Digital Repository and can be accessed at 10.5061/dryad.280gb5mrk (Besser, 2022).

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