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Methods in Ecology and Evolution

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Running Head: Primary producer amino acid δ^{15}N variability

Meta-analysis of primary producer amino acid δ^{15}N values and their influence on trophic position estimation

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Abstract

1. Compound-specific stable isotope analysis of individual amino acids (CSIA-AA) has emerged as a transformative approach to estimate consumer trophic positions (TP_{CSIA}) that are internally indexed to primary producer nitrogen isotope baselines. Central to accurate TP_{CSIA} estimation is an understanding of beta (β) values—the differences between trophic and source AA $\delta^{15}N$ values in the primary producers at the base of a consumers' food web. Growing evidence suggests higher taxonomic and tissue-specific β value variability than typically appreciated.

2. This meta-analysis fulfills a pressing need to comprehensively evaluate relevant sources of β value variability and its contribution to TP_{CSIA} uncertainty. We first synthesized all published primary producer AA $\delta^{15}N$ data to investigate ecologically relevant sources of variability (e.g., taxonomy, tissue type, habitat type, mode of photosynthesis). We then reviewed the biogeochemical mechanisms underpinning AA $\delta^{15}N$ and β value variability. Lastly, we evaluated the sensitivity of TP_{CSIA} estimates to uncertainty in mean $\beta_{Glx-Phe}$ values and Glx-Phe trophic discrimination factors ($TDF_{Glx-Phe}$).

3. We show that variation in $\beta_{Glx-Phe}$ values is two times greater than previously considered, with degree of vascularization, not habitat type (terrestrial vs. aquatic), providing the greatest source of variability (vascular autotroph = -6.6 ± 3.4‰; non-vascular autotroph = $+3.3 \pm 1.8$‰). Within vascular plants, tissue type secondarily contributed to $\beta_{Glx-Phe}$ value variability, but we found no clear distinction among C_3, C_4, and CAM plant $\beta_{Glx-Phe}$ values. Notably, we found that vascular plant $\beta_{Glx-Lys}$ values ($+2.5 \pm 1.6$‰) are considerably less variable than $\beta_{Glx-Phe}$ values, making Lys a useful AA tracer of primary production sources in terrestrial systems. Our multi-trophic level sensitivity analyses demonstrate that TP_{CSIA} estimates are highly sensitive to changes in both $\beta_{Glx-Phe}$ and $TDF_{Glx-Phe}$ values but that the relative influence of β values dissipates at higher trophic levels.

4. Our results highlight that primary producer β values are integral to accurate trophic position estimation. We outline four key recommendations for identifying, constraining, and accounting for β value variability to improve TP_{CSIA} estimation accuracy and precision moving

forward. We must ultimately expand libraries of primary producer AA δ^{15}N values to better understand mechanistic drivers of β value variation.

Keywords: amino acid; autotroph; beta value; compound-specific isotope analysis; ecogeochemistry; food web; nitrogen stable isotope; trophic ecology

Background

Knowledge of an organism's position in a food web is foundational to understanding the structure and function of ecological communities (Leibold et al., 1997; Persson, 1999; Post et al., 2000). Characterizing the nitrogen isotope variation at the base of the food web ($\delta^{15}N_{baseline}$) remains one of the biggest challenges to accurately estimating consumer trophic position using stable isotope analysis (SIA). Over the past decade, compound-specific isotope analysis of amino acids (CSIA-AA) has transformed our ability to study food web dynamics as it allows for the simultaneous reconstruction of $\delta^{15}N_{baseline}$ and consumer trophic ecology without needing to independently identify and isotopically characterize the relative contribution of $\delta^{15}N_{baseline}$. Trophic dynamic studies using CSIA-AA are premised on differential isotopic fractionation of individual AAs during trophic transfer (McMahon & McCarthy, 2016), with AAs broadly categorized as those that do ("trophic" AA) and do not ("source" AA) undergo significant trophic fractionation (Popp et al., 2007). Importantly, because the $\delta^{15}N$ values of source AAs (phenylalanine: Phe; methionine: Met; lysine: Lys; tyrosine: Tyr) remain relatively unaltered moving through the food web, they provide an internally indexed estimate of $\delta^{15}N_{baseline}$. These data, when used in combination with the $\delta^{15}N$ values of trophic AAs [glutamic acid (Glu) + glutamine (Gln): Glx; aspartic acid (Asp) + asparagine (Asn): Asx; alanine: Ala; isoleucine: Ile; leucine: Leu; proline: Pro; valine: Val], provide a method for estimating consumer trophic positions (TP_{CSIA}) calibrated to each consumer's integrated $\delta^{15}N_{baseline}$. This ability to simultaneously partition isotopic variability resulting from changes in $\delta^{15}N_{baseline}$ versus changes in the number of trophic transfers has been used to reveal foraging patterns of cryptic species and systems (Gerringer et al., 2017; Saccò et al., 2019), reconstruct ancient diets and food webs (Jarman et al., 2017; McMahon et al., 2019), and resolve complex trophic dynamics and patterns in animal movement (Dale et al., 2011; Matsubayashi et al., 2020).

Understanding of AA-specific trophic discrimination factors (TDFs) and primary producer $\delta^{15}N$ values is fundamental to accurate trophic position estimation using CSIA-AA (Figure 1). TP_{CSIA} is most commonly estimated using the equation:

$$TP_{CSIA} = 1 + \frac{(\delta^{15}N_{Trophic\ AA} - \delta^{15}N_{Source\ AA} - \beta)}{TDF_{Trophic\ AA - Source AA}}$$
(1)

where $\delta^{15}N_{Trophic\ AA}$ and $\delta^{15}N_{Source\ AA}$ represent the $\delta^{15}N$ values of consumer trophic and source AAs (typically Glx and Phe, respectively); $TDF_{Trophic\ AA–Source\ AA}$ is the trophic discrimination factor

reflecting changes in trophic and source AA δ^{15}N values between diet and consumer ($TDF_{\text{Trophic AA–Source AA}} = \Delta^{15}N_{\text{Trophic AA}} - \Delta^{15}N_{\text{Source AA}}$); and β (beta) is the difference between trophic and source AA δ^{15}N values in the primary producer(s) at the base of the food web. A confluence of evidence suggests variability in AA-specific TDFs within and among taxa is mechanistically linked to variation in animal physiology and biochemistry (Bradley et al., 2015; McMahon & McCarthy, 2016; Nielsen et al., 2015), with diet quality and mode of nitrogen excretion emerging as major drivers of variability (Germain et al., 2013; McMahon, Thorrold, et al., 2015). Similarly, seminal research identified characteristic differences in β values among different groups of primary producers—algae, C_3 plants, and C_4 plants—that are now routinely applied in trophic ecology studies (Chikaraishi et al., 2009, 2010). Although research into and consideration of AA-specific TDF variability continues to grow (Figure 2), study of β values, including sources of heterogeneity and influence on TP_{CSIA}, has lagged far behind.

Through integration of data from cultivated and wild primary producers, Chikaraishi et al. (2009, 2010) first identified what have become conventionally applied β values for aquatic algae (mean ± SD: +3.4 ± 0.9‰) and vascular plants (C_3: −8.4 ± 1.6‰, C_4: −0.4 ± 1.7‰). However, multiple recent studies have demonstrated there can be substantial AA δ^{15}N value variation within these groups that can influence β values, particularly among terrestrial plants. For example, Kendall et al. (2019) identified an ~3.5‰ difference in β values between woody and herbaceous C_3 plants, and differential isotopic fractionation of Phe appears to also contribute to differences in β values among mangrove species (Smallwood et al., 2003) and among some grass tissues (Bol et al., 2002; Styring et al., 2014). Takizawa & Chikaraishi (2017) and Takizawa et al. (2017) demonstrated that the phenology of plant tissue synthesis can strongly influence tissue-specific primary producer AA δ^{15}N values, with early growth leaves and flowers having different isotopic compositions and β values than those generated later in the season. McCarthy et al. (2013) observed differences in AA δ^{15}N values within and among cultured eukaryotic microalgae and cyanobacteria, which may yield divergent β values for these taxa. Such currently underappreciated isotopic and β value variation within and among primary producers holds the potential to strongly impact TP_{CSIA} estimates.

Given the rise in application of CSIA-AA to trophic ecology studies, there is a pressing need to re-visit β to identify ecologically relevant sources of variability and evaluate the influence of this

variability on TP_{CSIA} estimates. Herein, we first performed a comprehensive meta-analysis of published primary producer AA $\delta^{15}N$ data, which were then used to re-evaluate variability in β values among ecological systems, taxonomic groups, producer tissues, and modes of photosynthesis. We identify and discuss likely biogeochemical mechanisms underpinning this variation. We then evaluate the influence of variation in mean $\beta_{Glx-Phe}$ (and $TDF_{Glx-Phe}$) values on TP_{CSIA} estimates by performing sensitivity analyses that use published consumer AA $\delta^{15}N$ data within three systems with divergent primary producer communities: (1) a relatively simple terrestrial insect food web containing only vascular plants; (2) a more complex freshwater aquatic food web containing algae, aquatic plants, and terrestrial plants; and (3) a relatively simple pelagic open ocean food web containing only microalgae and cyanobacteria. Lastly, we provide four key recommendations for identifying, constraining, and accounting for β value variability to improve TP_{CSIA} estimation accuracy and precision moving forward, including highlights of critical areas of future research.

Variability in Primary Producer Amino Acid Nitrogen Isotope Ratios

A comprehensive literature search of primary producer AA $\delta^{15}N$ values yielded 51 studies from which 450 β values could be estimated for individual primary producers (see Supporting Information for detailed methods). To reduce sampling bias, we used simple means of species-specific tissues within studies as our unit of replication. This process resulted in a final dataset that consisted of 236 β values across $\geq$ 132 different primary producer genera (Table 1, Figure 3). Our discussion focuses primarily on β values derived from Glx and Phe ($\beta_{Glx-Phe}$) because they are the most commonly measured and applied trophic and source AAs for estimating TP_{CSIA}. However, we also present β values for all trophic (Asx, Ala, Ile, Leu, Pro, Val) and "metabolic" (Thr) AAs relative to all source AAs (Phe, Lys, Met, Tyr) with discussion of potential alternative useful pairings wherever pertinent (Tables 2, S1; Figures 3, S1). These data come from a mix of natural and human manipulated primary producers (e.g., laboratory or farm settings; Table 1), which may contribute to β value variation in some taxa dependent on N source and N assimilation pathway (see *Insights into β Variability: Nitrogen Assimilation and Amino Acid Biosynthesis*; Figure S2). Marine phytoplankton data were primarily from laboratory cultures whereas macroalgal, seagrass, and freshwater primary producer data were

primarily from natural environments. Approximately two-thirds of the terrestrial plant data were from cultivated plants in suburban/urban or farm settings.

We found β values varied substantially across taxonomic groups and tissue types (Figures 3, S1), with degree of vascularization, regardless of habitat type (terrestrial, freshwater, marine), providing the single greatest source of variability. The $\beta_{Glx-Phe}$ patterns for non-vascular autotrophs, including aquatic algae, cyanobacteria, chemoautotrophic bacteria, and moss (mean ± SD: +3.3 ± 1.8‰, n = 68; Table 2), generally aligned with those reported by Chikaraishi et al. (2009) (+3.4 ± 0.9 ‰). However, $\beta_{Glx-Phe}$ values for vascular plants (–6.6 ± 3.4‰, n =152; Table 2) were distinctly higher than those reported by Chikaraishi et al. (2010). Moreover, our meta-analysis revealed that $\beta_{Glx-Phe}$ value standard deviations of non-vascular (±1.8 ‰) and vascular (±3.4 ‰) autotrophs were two times larger than the values conventionally applied (non-vascular = ±0.9‰, vascular = ±1.6‰) to estimate TP_{CSIA} (Figure 3, Table 2). In contrast to Chikaraishi et al. (2010), we did not find mode of photosynthesis (C_3, C_4, CAM) to be the primary contributor to β value variability within terrestrial plants. β values derived using the other trophic AAs and Phe (β_{X-Phe}) produced similar patterns of means and variance within and among taxonomic groups (Figure S1, Tables 2, S1). Notably, we observed that β values derived using Lys as the source AA (β_{X-Lys}) were remarkably well-constrained across vascular plants relative to β_{X-Phe} values (e.g., $\beta_{Glx-Lys}$ = +2.5 ± 1.6‰, $\beta_{Glx-Phe}$ –6.6 ± 3.4‰; Tables 2, S1). In the subsequent subsections we explore these patterns of β value variability within and among: (1) vascular autotrophs (i.e., true plants), both terrestrial and aquatic, and (2) non-vascular autotrophs, including eukaryotic microalgae and macroalgae, cyanobacteria, and chemoautotrophic bacteria.

Vascular Primary Producers

True plants, by far the most analyzed group of primary producers (65.2 % of analyzed taxa), had distinct $\beta_{Glx-Phe}$ values from non-vascular primary producers, regardless of habitat type (terrestrial, freshwater, marine; Table 1). To allow for comparisons within and among groups of vascular plants, we categorized data by broad taxonomic group (fern, cactus, forb, grass, vine, shrub, tree, seagrass, macrophyte), stem type (herbaceous: forb, grass; woody: vine, shrub, tree), tissue type (e.g., leaf, shoot, seed), tissue class (leaf, structural, reproductive), and mode of photosynthesis (C_3, C_4, CAM).

We then used non-parametric Wilcoxon rank-sum tests to statistically compare β values among vascular plants based on stem type, tissue class, and mode of photosynthesis. Statistical comparisons were restricted to plant groupings with sample sizes ≥ 5, which necessarily limited quantitative analyses to well-studied groups of true plants (forbs, grasses, trees). In this paper we present data and statistical parameters to discuss observed patterns but do not assign statistical significance as per the recommendation of Hurlbert et al. (2019).

Our meta-analysis lends support to the notion that lignin biosynthesis is a key driver of $\beta_{Glx-Phe}$ value variability within terrestrial plants. Following Kendall et al. (2019), we found $\beta_{Glx-Phe}$ values (mean ± SD) were higher for herbaceous (forbs, grasses; –5.2 ± 3.6‰, $n = 66$) versus woody (vine, shrub, tree; –7.7 ± 2.9‰, $n = 76$) plants (Wilcoxon rank-sum tests: $W = 1330$, $P < 0.001$). This pattern was maintained across all β_{X-Phe} combinations, with mean differences between herbaceous and woody plants ranging between 2.2‰ ($\beta_{Pro-Phe}$) and 4.1‰ ($\beta_{Asx-Phe}$). $\beta_{Glx-Lys}$ values were similarly higher for herbaceous (+3.3 ± 1.1‰, $n = 27$) versus woody (+1.9 ± 1.5‰, $n = 29$) plants (Wilcoxon rank-sum tests: $W = 225$, $P = 0.002$), but mean differences were generally narrower across β_{X-Lys} values relative to β_{X-Phe} values ranging between 0.4‰ ($\beta_{Val-Lys}$) and 2.9‰ ($\beta_{Ile-Lys}$). (Figures 3, S1). The contribution of lignin production to β value variation is further qualitatively supported by the lone datum for a bryophyte (moss), which lacks lignin and had a β value intermediate to vascular and non-vascular primary producers (Figure 3). Further study of nonvascular terrestrial plants will shed important light on relationships among lignin biosynthesis, AA nitrogen isotope fractionation, and β value variability.

We observed variation in β values among certain terrestrial plant tissues, which may be driven in part by differences in plant N assimilation pathway. $\beta_{Glx-Phe}$ values were higher in reproductive tissues (seeds, fruits, and flowers) relative to leaf tissues in forbs (reproductive: –2.6 ± 3.9‰, $n = 7$; leaf: –7.1 ± 2.5‰, $n = 30$; $W = 49$, $P = 0.016$) and woody plants (reproductive: –4.3 ± 4.8‰, $n = 14$; leaf: –8.5 ± 1.6‰, $n = 59$; $W = 194$, $P = 0.002$). However, within forbs, these differences primarily reflect variation within N_2-fixing plants whose tissue-specific $\beta_{Glx-Phe}$ values differed to a much greater extent (reproductive: +0.2 ± 0.9‰, $n = 5$; leaf: –6.5 ± 2.1‰, $n = 4$) than those of other forbs (reproductive: –6.3 ± 3.0‰, $n = 3$; leaf: –7.2 ± 2.5‰, $n = 28$). Samples sizes were small and thus inferences were limited. Nevertheless, this is suggestive of interactive effects of N assimilation pathway and tissue biosynthesis on $\beta_{Glx-Phe}$ values and warrants further evaluation. Collectively, these

tissue-specific differences in β values may uniquely influence TP_{CSIA} estimates for species that specialize on seeds or fruits, as well as their predators. In contrast, limited data suggests $\beta_{Glx-Phe}$ values were generally similar among grass tissues (Figures 3, S1), although β values that include the trophic AA Ile ($\beta_{Ile-Phe}$, $\beta_{Ile-Lys}$, $\beta_{Ile-Tyr}$) appear higher for grass structural tissues relative to both reproductive and leaf tissues (Figure S1A, B, D). These forb, grass, and woody plant tissue-specific β value patterns extended to most β_{X-Phe} combinations that could be evaluated (Figure S1); there were generally insufficient data to evaluate tissue-specific β_{X-Lys}, β_{X-Tyr}, and β_{-Met} variation. While tempting to speculate about potential underlying mechanisms, a necessary limitation of this meta-analysis is that it required pooling of limited data from a variety of taxa across space and time and is thus not adequately designed to robustly evaluate all potential sources of variability. Our understanding of tissue-specific β value variation would thus be enhanced through more comprehensive sampling of multiple tissue types from the same individuals or species. Similarly, more targeted experimental studies would allow for quantitative evaluation of how suites of factors (e.g., vascularity, lignin production, N assimilation pathway, AA concentrations, AA turnover rates) interact to influence β values.

Our meta-analysis suggests that mode of photosynthesis (C_3, C_4, CAM) is not the primary driver of β value variability within terrestrial plants. Chikaraishi et al. (2010) was the first to suggest divergent $\beta_{Glx-Phe}$ values for C_3 and C_4 food webs, observing distinct differences between cultivated terrestrial C_3 (–8.4 ± 1.6‰, n = 17) and C_4 (–0.4 ± 1.7‰, n = 5) plants. While we similarly observed elevated $\beta_{Glx-Phe}$ values for C_4 (–1.2 ± 2.8‰, n = 11) relative to C_3 plants (–6.9 ± 3.2‰, n = 133)—likely due in large part due to limited additional sampling of C_4 plants—our expanded dataset demonstrates C_4 plant $\beta_{Glx-Phe}$ values fall well within the bounds of variability of C_3 plants (Figure 3). In fact, observed differences in $\beta_{Glx-Phe}$ values between C_3 and C_4 plants appear to be driven by variability in analyzed grass leaves (C_4: +0.5 ± 1.7‰, n = 7; C_3: –6.9 ± 3.0‰, n = 4; W = 87.5, $P <$ 0.001). $\beta_{Glx-Phe}$ values appear similar for C_3 and C_4 grass seeds (C_4: –5.1 ± 0.5‰, n = 2; C_3: –6.0 ± 1.8‰, n = 6) and shoots (C_4: –1.2‰, n = 1; C_3: –3.9 ± 3.5‰, n = 8), and C_3 (–6.3 ± 3.3‰, n = 37) and C_4 (–5.0‰, n = 1) forbs (Figure 3). Additionally, geographically constrained studies have shown that $\beta_{Glx-Phe}$ values can be similar among co-occurring plants with divergent modes of photosynthesis. For example, similar $\beta_{Glx-Phe}$ values have been observed for C_3 (–5.8 ± 2.9‰; n = 10), C_4 (–5.0 ± 2.2‰; n

= 9), and CAM (-5.3 ± 2.3‰; $n = 5$) plants (forbs, grasses, cacti, shrubs) from an arid habitat in New Mexico (A. C. Besser, unpublished data), and Ostle et al. (1999) observed similar $\beta_{Glx\text{-}Phe}$ values for C_4 (maize: -1.2‰) and C_3 (winter wheat: -2.0‰) grasses grown in experimental plots in France. Ultimately, given that C_4 and CAM plants are heavily underrepresented in the CSIA-AA literature, further study will help clarify the potential role that mode of photosynthesis plays in β value variability.

Aquatic vascular plants are also understudied. Reported seagrass $\beta_{Glx\text{-}Phe}$ values (range: -8.7 to -6.6‰, $n = 6$) were similar to those of other vascular plants (Figure 3), likely due to their evolution from a single lineage of terrestrial flowering plants (Waycott et al., 2006). Available data suggest seagrass β values may be significantly less variable than other vascular autotrophs. However, only two (*Thalassia testudinum*, *Zostera marina*) of ~60 extent seagrass species have been analyzed for their AA $\delta^{15}N$ values, so whether this observation is an effect of limited sampling or related to their relatively conservative evolutionary pathway warrants further evaluation (Short et al., 2007). $\beta_{Glx\text{-}Phe}$ values for freshwater vascular plants (macrophytes) were similar to those of marine and terrestrial plants (Figure 3). However, quantitative comparisons were not possible due to small sample sizes. Further refinement of marine and freshwater vascular plant β values will help constrain future applications of CSIA-AA to trophic ecology studies in freshwater and coastal habitats; see section "*Recommendations for Refining Beta Values*".

Non-Vascular Primary Producers

$\beta_{Glx\text{-}Phe}$ values of non-vascular primary producers were remarkably similar among the diverse group of distantly related taxa, including eukaryotic microalgae ($+3.9 \pm 1.4$‰, $n = 27$; marine and freshwater), macroalgae ($+3.5 \pm 0.7$‰, $n = 14$), cyanobacteria ($+2.1 \pm 2.2$‰, $n = 19$), and chemoautotrophic bacteria ($+4.5 \pm 1.9$‰, $n = 4$; Figure 3). This follows general patterns first observed by Chikaraishi et al. (2009) and McCarthy et al. (2013). Cyanobacteria $\beta_{Glx\text{-}Phe}$ values were lower than eukaryotic microalgae (Wilcoxon rank-sum test: $W = 136.5$, $P = 0.034$), but macroalgae $\beta_{Glx\text{-}Phe}$ values were similar to both cyanobacteria and eukaryotic microalgae ($P > 0.05$). However, despite this general similarity among groups of non-vascular primary producers, we still observed higher variation in $\beta_{Glx\text{-}}$

Phe values (SD = 1.8‰) than previously considered for aquatic photoautotrophs (SD = 0.9‰; Chikaraishi et al. 2009; Table 2).

Marine non-vascular primary producers were the second-most analyzed group of autotrophs in our meta-analysis (30.3 % of analyzed taxa; Table 1). We found that $\beta_{X\text{-Phe}}$ values were generally similar among green (n = 3), brown (n = 7), and red (n = 4) macroalgae and among cyanobacteria orders Nostocales (n = 7), Oscillatoriales (n = 5), and Synechococcales (n = 7; Wilcoxon rank-sum tests; P > 0.05). However, there may be biologically meaningful differences among some groups of marine eukaryotic microalgae. McCarthy et al. (2013) were the first to suggest that some algal lineages may have unique physiological characteristics that yield distinct AA $\delta^{15}N$ patterns (i.e., isotope 'fingerprints'), specifically _Prochlorococcus marinus_ versus other cyanobacteria. Although sample sizes were small, our findings suggest diatoms (+2.3 ± 1.2‰, n = 5) have lower $\beta_{Glx\text{-Phe}}$ values than chlorophytes (+4.5 ± 1.6‰; n = 10; W = 44, P = 0.019) and possibly dinoflagellates (+4.3 ± 0.5‰, n = 4; W = 18, P = 0.063; Figure S3). Diatoms have unique physiologies and metabolisms, such as silica-lined cell walls, a central vacuole that stores nutrients, and functional urea uptake and utilization pathways (Bromke, 2013; Falkowski et al., 2004; Tozzi et al., 2004), which may contribute to differences in AA $\delta^{15}N$ patterns relative to other phytoplankton. Of note, almost all β values for eukaryotic microalgae and cyanobacteria to date were derived from laboratory cultures. Although methodologically challenging, further study of wild-collected phytoplankton taxa is needed to better assess natural β value variability.

Freshwater non-vascular primary producers are woefully understudied within the CSIA-AA literature (3.8 % of analyzed taxa; Table 1), with only four studies reporting AA $\delta^{15}N$ data (Fogel & Tuross, 1999; Ishikawa et al., 2014; Ohkouchi et al., 2015; Zhang et al., 2019, 2021). Freshwater eukaryotic microalgae $\beta_{Glx\text{-Phe}}$ values (+4.2 ± 0.7‰, n = 5) were similar to those of their marine counterparts (+3.9 ± 1.6‰, n = 22, P > 0.05; Figure 3). Lack of taxonomic identifications prevented further analysis of the limited freshwater non-vascular autotroph data but this is an area of important future research given the rapid growth in TP$_{CSIA}$ applications in freshwater systems.

Chemoautotrophs, which derive energy from the oxidation of inorganic compounds, are among the least characterized groups within the AA $\delta^{15}N$ literature. Data for chemoautotrophic bacteria in this meta-analysis were derived from just two studies, Pan et al. (2007) and Yamaguchi et

al. (2017). Notably, chemoautotroph $\beta_{Glx-Phe}$ values were similar to those of the much more studied eukaryotic microalgae and cyanobacteria groups, which may be because the analyzed microbes all use the phenylpyruvate pathway for Phe synthesis (Yamaguchi et al. 2008). Nevertheless, chemoautotrophs exhibit considerable physiological and phylogenetic diversity, which may ultimately influence AA biosynthesis and β values within this diverse group of organisms (Nakagawa & Takai, 2008). Given their importance to food webs in extreme environments like hydrothermal vents, cold seeps, natural gas and methane seeps, and anoxic sediment waters (Nakagawa & Takai, 2008), expanded investigation of nitrogen isotope dynamics associated with chemoautotroph AA biosynthesis will improve food web studies within these systems.

Insights into β Variability: Nitrogen Assimilation and Amino Acid Biosynthesis

In this section, we discuss nitrogen assimilation and AA biosynthesis and degradation pathways to highlight the potential mechanisms leading to variation in β values within and among primary producer groups. We focus primarily on terrestrial plants because they are the most analyzed group of autotrophs and exhibit the largest range in β values. However, also make note that many of the processes discussed are directly applicable to non-vascular autotroph nitrogen metabolism. Generally, variation in the most often employed β values (e.g., $\beta_{Glx-Phe}$ and $\beta_{Glx-Lys}$) can likely be attributed to variability in the $\delta^{15}N$ values of source AAs due to their catabolism for secondary metabolite synthesis within primary producers. In contrast, trophic AAs likely have less variable $\delta^{15}N$ values within primary producers due to their more central role in nitrogen metabolism. This pattern is opposite to that commonly found in consumers, where trophic AAs exhibit most of the $\delta^{15}N$ variability (McMahon & McCarthy, 2016).

Autotrophs acquire and assimilate nitrogen using multiple complex pathways (Figure 4). The extent to which autotrophs utilize each of the various nitrogen sources (e.g., NO_3^-, NH_4^+, N_2, AA) depends on a number of factors, including taxonomy, growth state, microbial symbiont status, and environmental conditions (Jackson et al., 2008; Nacry et al., 2013; Szpak, 2014). These processes can contribute to variation in $\delta^{15}N_{baseline}$ (i.e., the bulk tissue $\delta^{15}N$ values of primary producers at the base of a food web) and individual AA $\delta^{15}N$ values because there are differential kinetic isotope effects associated with each inorganic nitrogen acquisition pathway (Lachmann et al., 2019; Werner &

Schmidt, 2002), such that bulk tissue $\delta^{15}N$ values of primary producers are different than the $\delta^{15}N$ values of their primary N source (Szpak, 2014). However, they likely do not strongly influence β values (i.e., the relative differences in $\delta^{15}N$ values among AAs within an individual) because both source and trophic AA $\delta^{15}N$ values are impacted similarly and once nitrogen is assimilated by the autotroph, it is transferred among molecules in a closed system (Werner & Schmidt, 2002). For example, cyanobacteria (*Synechococcus* sp., *Nostoc* sp., *Cyanothece* sp.) grown in the presence or absence of NO_3^- exhibit Glx and Phe $\delta^{15}N$ values that vary by 4.4–5.9‰ but $\beta_{Glx\text{-}Phe}$ values that vary by only 0.2–0.9‰ (Chikaraishi et al., 2009; McCarthy et al., 2013). Similarly, terrestrial plants (tomato fruit: *Solanum lycopersicum*; wheat seed: *Triticum* sp.) grown using various N sources (e.g., animal manure, synthetic fertilizers) exhibit more variable Glx and Phe values (tomato: 10.5 and 10.7‰, wheat: 9.6 and 11.0‰) than $\beta_{Glx\text{-}Phe}$ values (tomato: 2.4‰, wheat: 5.1‰; Bontempo et al., 2020; Paolini et al., 2015). However, there is some evidence that fertilizer application rates can slightly impact $\beta_{Asp\text{-}Phe}$ values (Paolini et al., 2015), but the effects of nitrogen supply rates on primary producer AA concentrations, AA $\delta^{15}N$ values, and β values have not been systematically tested, including in the above examples, and warrant further study.

There are two N assimilation pathways—direct uptake of AAs from the environment and N_2 fixation by microbial symbionts—that may contribute to variation in β values within and among taxa. Many autotrophs, including terrestrial plants, all bacteria, and some eukaryotic microalgae, have the capability to directly uptake AAs from their environment (i.e., soil or water; Kielland, 1994; Palenik & Morel, 1990; Zehr & Kudela, 2011). Given that $\delta^{15}N$ values of soil AAs are highly variable because they originate from a variety of organismal sources and undergo varying degrees of degradation before assimilation (Philben et al., 2018), differential rates of direct AA uptake could increase variation in β values among primary producers. The relative importance of this incorporation mechanism varies greatly and is more likely to occur in regions with high soil organic matter content and low decomposition rates (e.g., the Arctic tundra; Kielland, 1994) than in ecosystems with high decomposition and mineralization rates (but see Gioseffi et al., 2012). In addition, autotrophic symbioses with microbes that can access atmospheric nitrogen sources, particularly N_2 (e.g., plants and N_2-fixing *Rhizobium*, diatoms and N_2-fixing cyanobacteria), can uniquely influence Ala, Asp, Asn, Glu, and Gln production rates (Werner & Schmidt, 2002; Lambers et al., 2008; Liu et al., 2018)

and likely their $\delta^{15}N$ values (Figure 4). This differential impact of low $\delta^{15}N_{baseline}$ values on select AAs could potentially impact variation in β values, although this has not been systematically explored.

The $\delta^{15}N$ values of the source AAs Phe and Lys are likely a major source of variation in β values. Both Phe and Lys, which receive amine groups from Glu during their biosynthesis from chorismate and Asp, likely undergo significant isotopic fractionation during their catabolism into precursors of numerous important secondary compounds which could lead to variation in β values (Galili et al., 2001; Kendall et al., 2019). The extent to which Phe and Lys are catabolized for secondary compound synthesis varies due to a variety of factors, such as growth form and environmental conditions (Galili et al., 2001; Sharma et al., 2019). Phe may display greater isotopic fractionation than Lys and thus a wider range in $\delta^{15}N$ values as it is typically catabolized more frequently; approximately a third of plant organic matter is synthesized from Phe (Maeda & Dudareva, 2012; Pascual et al., 2016). Phe is a precursor to many secondary compounds, including lignin, flavonoids, and tannins (Maeda & Dudareva, 2012; Vogt, 2010). Kendall et al. (2019) reported a positive correlation between the concentrations of lignin—a structural compound—and Phe $\delta^{15}N$ values. This relationship occurs because the first step in Phe catabolism for the synthesis of secondary metabolites is deamination, in which Phe loses an amine group to become cinnamate (Deng & Lu, 2017; Kendall et al., 2019). This initial deamination step exhibits isotopic discrimination (Hermes et al., 1985), such that Phe molecules containing ^{14}N are preferentially deaminated, leaving behind a ^{15}N-enriched residual pool of Phe $\delta^{15}N$ values (Kendall et al., 2019). Thus, plants composed of more lignin, like trees, typically have relatively higher Phe $\delta^{15}N$ values (and thereby lower $\beta_{Glx-Phe}$ values) than herbaceous plants containing less lignin (Kendall et al., 2019). Lys, on the other hand, can act as an alternative respiratory substrate, increasing in concentration during periods of abiotic stress, and is also a precursor to metabolites involved in plant immunity (Galili et al., 2001; Zeier, 2013). Recent work on a freshwater green alga and terrestrial C_3, C_4, and CAM plants found that Lys $\delta^{15}N$ values were lower and less variable than those of Phe across producer groups and were strongly correlated to bulk tissue $\delta^{15}N$ values (A. C. Besser, unpublished data), a pattern that likely explains the low variability in β_{X-Lys} values relative to β_{X-Phe} values observed herein (Figures 3, S1). Phe $\delta^{15}N$ values did not correlate with bulk tissue $\delta^{15}N$ values in these producer groups, highlighting the highly variable nature of Phe $\delta^{15}N$ values.

In contrast to Phe and Lys, Glu and Gln are unlikely to contribute significantly to β value variability. These AAs are central to plant nitrogen metabolism and have high turnover rates, particularly in leaves (Kruse et al., 2003). During assimilation, plants add NH_4^+, reduced from NO_3^- and NO_2^- via nitrate reductase and nitrite reductase, respectively (Werner & Schmidt, 2002), to an α-ketoglutarate molecule using glutamate dehydrogenase to form Glu or an existing Glu molecule using glutamine synthase to form Gln (Werner & Schmidt, 2002; Lambers et al., 2008). Once assimilated, nitrogen is primarily shuttled among compounds within plant tissues via Glu and Gln (Figure 4), which donate amine groups during AA biosynthesis via transamination reactions (Werner & Schmidt, 2002). As a result, Glu and Gln, which make up the highest proportion of AAs in plant proteomes (Hildebrandt et al., 2015), are likely isotopically well-mixed within the internal nitrogen pool, potentially leading to lower variability in their $\delta^{15}N$ values as compared to other AAs.

Lastly, tissue-specific differences in plant trophic or source AA $\delta^{15}N$ values, and by extension β values, likely occur due to differing AA concentrations and turnover rates among plant tissue types (Camargos et al., 2004; Kruse et al., 2003; Mapelli et al., 2001). Differences in AA concentrations are largely determined by free, or soluble, AA concentrations, which vary greatly with changing nitrogen supply conditions (Caputo & Barneix, 1997), rather than changes in protein-bound AA concentrations. For example, Glu and Gln are among the most abundant free and protein-bound AAs in *Canavalia ensiformes* seeds and leaf tissues, but among the least abundant AAs in seedling plant stem tissues (Camargos et al., 2004). Phe displays the opposite pattern, being most abundant in seedling plant stem tissues but least abundant in leaf and seed tissues (Camargos et al., 2004). AAs, and thereby AA $\delta^{15}N$ values, typically turnover more quickly in leaves than in other tissues and in younger tissues than in older tissues (Kruse et al., 2003). Different AA turnover rates among tissue types may lead to significant differences in AA $\delta^{15}N$ values and, in turn, more variable β values among tissue types. However, more experimental studies measuring plant AA concentrations, turnover rates, and $\delta^{15}N$ values among tissue types are needed to constrain the relative magnitudes of variation.

Sensitivity of Trophic Position Estimates to Uncertainty in Beta Values

Given our observation of much larger variation in $\beta_{Glx\text{-}Phe}$ values than typically appreciated (but see O'Connell & Collins, 2018), we evaluated how assumptions of mean β values and TDFs influence TP_{CSIA} estimates. To this end, we performed bivariate sensitivity analyses to estimate and compare TP_{CSIA} for 11 consumers within three model systems (terrestrial, freshwater, oceanic) using published consumer $\delta^{15}N_{Glx}$ and $\delta^{15}N_{Phe}$ data and all combinations of $\beta_{Glx\text{-}Phe}$ and $TDF_{Glx\text{-}Phe}$ values within ecologically informed ranges as input parameters (i.e., we estimated TP_{CSIA} for each consumer using many combinations of assumed $\beta_{Glx\text{-}Phe}$ and $TDF_{Glx\text{-}Phe}$ values that span their known ecological ranges). These model systems were chosen to reflect scenarios where only vascular (terrestrial), only non-vascular (oceanic), or both vascular and non-vascular (freshwater) primary producer assemblages support focal food webs. System- and consumer-specific $\beta_{Glx\text{-}Phe}$ value ranges were derived from our meta-analysis (Figure 3), whereas $TDF_{Glx\text{-}Phe}$ ranges were derived from variation related to consumer and system type, including reductions due to shifts in diet quality and/or mode of nitrogen excretion (see meta-analysis by McMahon & McCarthy, 2016).

In the terrestrial case study, we modeled TP_{CSIA} for four consumers within an insect food web on a mature apple orchard (TP 2: apple aphid, *Aphis pomi*; TP 3: hoverfly, *Eupeodes sp.*; TP 4: parasitoid wasp, *Bothriothorax sp*; TP 5: hyperparasitoid wasp, *Pachyneuron albutius*; Steffan et al., 2013). Only vascular primary producers contribute nitrogen to this food web. We used the Chikaraishi et al. (2009) single-TDF equation in combination with the published consumer $\delta^{15}N_{Glx}$ and $\delta^{15}N_{Phe}$ data to estimate consumer TP_{CSIA} for all combinations of ecologically-relevant $\beta_{Glx\text{-}Phe}$ values between –12.0 and 0.0‰ from this study and $TDF_{Glx\text{-}Phe}$ values between 6.5 and 8.5‰ (mean = 7.5‰) as observed for terrestrial insects (e.g., Chikaraishi et al., 2010, 2011; Steffan et al., 2013; Takizawa et al., 2020).

In the freshwater case study, we modeled TP_{CSIA} for three consumers within a hypothetical freshwater food web where both vascular and non-vascular primary producers contribute nitrogen to the system (TP 2: caddisfly, Hydropsychidae sp.; TP3: trout, *Oncorhynchus masou ishikawae*; TP 4: osprey, *Pandion haliaetus*; Elliott et al., 2021; Ishikawa et al., 2014). For this example, we used the McMahon & McCarthy (2016) multi-TDF equation to estimate consumer TP_{CSIA} using $TDF_{Glx\text{-}Phe}$ ranges of 6.5 to 8.5‰ (mean = 7.5‰) for low trophic level, ammonia-producing primary (insect) and secondary (fish) consumers (e.g., Bowes and Thorp, 2015; Blanke et al., 2017) and 3.5 to 5.5‰ (mean

= 4.5‰) for the carnivorous, uric acid-producing tertiary consumer (osprey; McMahon, Polito, et al., 2015; Hebert et al. 2016; Elliott et al., 2021). $\beta_{Glx-Phe}$ values were between −12 and 6‰ that spanned the full vascular to non-vascular $\beta_{Glx-Phe}$ value range from this meta-analysis.

In the oceanic case study, we modeled TP_{CSIA} estimates for four consumers from the oceanic North Pacific Subtropical Gyre, an oligotrophic system where only non-vascular autotrophs (eukaryotic microalgae and cyanobacteria) drive primary production (TP 2: copepod, *Oithona* sp.; TP 3: flying fish, *Exocoetus volitans*; TP 4: yellowfin tuna, *Thunnus albacares*; TP 5: swordfish, *Xiphias gladius*; Hannides et al., 2009; Choy et al., 2015). For this example, we used the McMahon & McCarthy (2016) multi-TDF equation to estimate consumer TP_{CSIA} using $TDF_{Glx-Phe}$ ranges of 6.5 to 8.5 ‰ (mean = 7.5 ‰) for low trophic level, ammonia-producing primary (copepod) and secondary (flying fish) consumers (e.g., Bowes & Thorp, 2015; Blanke et al., 2017) and 4.5 to 6.5 ‰ (mean = 5.5 ‰) for the carnivorous tertiary and quaternary consumers (tuna and swordfish) (Bradley et al. 2015; Choy et al. 2015; McMahon, Thorrold et al. 2015), as well as $\beta_{Glx-Phe}$ values between 0 and 6 ‰ from this meta-analysis.

To allow for quantitative assessments of how variation in input parameters ($\beta_{Glx-Phe}$ and $TDF_{Glx-Phe}$) influence TP_{CSIA} estimates within and among consumers in our case studies, we normalized true TP_{CSIA} estimates to a baseline TP_{CSIA} estimate (i.e., ΔTP_{CSIA} = raw TP_{CSIA} − baseline TP_{CSIA}). For each consumer, the baseline TP_{CSIA} estimate was that derived from the mean TDF (7.5 ‰ for primary and secondary consumers, 5.5 ‰ for 3°+ fish consumers, or 4.5 ‰ for 3°+ bird consumers) and a β value of −6.50 (terrestrial), −1.75 (freshwater), or +3.25 (oceanic). These β values approximate the mean vascular (−6.50) and non-vascular (+3.25) β values resulting from this meta-analysis or their mean (−1.75), assuming a mix of 50 % vascular and 50 % non-vascular autotroph contribution (Table 3). Resulting ΔTP_{CSIA} estimates were binned by quarter trophic position for each consumer (Figure 5).

Our analyses revealed that TP_{CSIA} estimates were highly sensitive to both univariate and bivariate changes in $\beta_{Glx-Phe}$ and $TDF_{Glx-Phe}$ values (Figure 5, Table 3), and that sensitivity varied as a function of consumer and system type. Across all three case studies there was a general pattern of increasing TP_{CSIA} sensitivity to both $\beta_{Glx-Phe}$ and $TDF_{Glx-Phe}$ values with increasing trophic position. This is evidenced by the narrowing of the space between each isocline (i.e., 0.25 TP bin) with each

trophic transfer, which indicates that changes in parameter values will lead to bigger changes in TP_{CSIA} estimates for higher order consumers relative to lower order consumers. For example, across the three case studies TP_{CSIA} estimates spanned 1.1–3.0 for primary consumers but 2.1–5.1 for the top predators (Table 3), illustrating that unconstrained variance in parameter values can yield 2+ unit variability in TP_{CSIA} estimates. Moreover, the magnitude of variability in TP_{CSIA} increased in relation to the range of possible $\beta_{Glx-Phe}$ values (Table 3). Combined, this work shows that constraining uncertainty in mean parameter values becomes increasingly important to accurate TP_{CSIA} estimation the further one samples up the food web.

The influence of $\beta_{Glx-Phe}$ values on TP_{CSIA} estimates relative to $TDF_{Glx-Phe}$ values generally dissipated up the food web. This is evidenced by the lowering of isocline slopes within the terrestrial and oceanic case studies with increasing trophic position (Figure 5A,C). This indicates that TP_{CSIA} estimates tend to become less sensitive to $\beta_{Glx-Phe}$ values (higher isocline slopes) and more sensitive to $TDF_{Glx-Phe}$ values (lower isocline slopes) higher up the food web. Mathematically, this pattern is unsurprising given that TDFs are applied multiple times in the denominator of the TP_{CSIA} equation (Eq. 1; i.e., once for each trophic transfer) but β values are only subtracted once in the numerator. However, it's important to note that for some lower order consumers there are areas in bivariate β–TDF value space where TP_{CSIA} estimates are more sensitive to changes in $\beta_{Glx-Phe}$ values than $TDF_{Glx-Phe}$ values (e.g., aphids where $\beta > -6.0$‰, caddisfly and trout where $\beta > +1.0$‰; Figure 5A,B).

Lastly, our sensitivity analysis revealed that assumptions of $TDF_{Glx-Phe}$ variability within food webs can have complex effects on the relative influence of β and TDF values on TP_{CSIA} estimates. This is illustrated by the abrupt shift in pattern of isocline slopes between TL 3 and 4 in the freshwater case study (Figure 5B), where we used an ecologically relevant lower $TDF_{Glx-Phe}$ value range (3.5–5.5‰) for osprey relative to the aquatic consumers (6.5–8.5‰) to reflect a coupled shift in diet quality and mode of nitrogen excretion. After this transition point, there are large areas in bivariate β–TDF value space where TP_{CSIA} estimates are very insensitive to changes in $TDF_{Glx-Phe}$ but highly sensitive to changes in $\beta_{Glx-Phe}$ value (e.g., β between –4.0 and +4.0). These results contrast sharply with those derived from a sensitivity analysis where we assumed $TDF_{Glx-Phe}$ was constant for all freshwatwer consumers (range: 5.5–7.5‰; Figure S4), which yields more predictable and consistent isocline patterns across consumers but at the expense of higher sensitivity of TP_{CSIA} estimates to

changes in β and TDF values (narrowed isocline bins). Collectively, these findings show that in some cases $\beta_{Glx-Phe}$ values are more influential to TP_{CSIA} estimation than $TDF_{Glx-Phe}$ values. However, CSIA-AA practitioners will likely rarely know *a priori* which parameter is more important, highlighting the need for more critical evaluation of both β and TDF values in addition to TP_{CSIA} equations (e.g., single- vs. multi-TDF) in all CSIA-AA trophic ecology studies.

Recommendations for Refining Beta Values

CSIA-AA has emerged as a powerful tool to study food web dynamics, yet fundamental questions remain about the ecogeochemical processes that drive variation in AA nitrogen isotope patterns at all levels of the food web. Our study demonstrates that β values are twice as variable as typically considered and that assumptions surrounding mean parameter values—both β and TDF—and how TDFs vary up the food chain can have complex effects on TP_{CSIA} estimates. As a result, both β and TDF values require greater scrutiny moving forward to improve estimated TP_{CSIA} accuracy and precision.

The use of CSIA-AA within trophic ecology studies has grown in popularity because (1) the realized $\delta^{15}N_{baseline}$ is internally indexed, reducing uncertainty associated with identifying and analyzing primary producer $\delta^{15}N$ values *a priori*; (2) β values, the difference in $\delta^{15}N_{TrophicAA}$ and $\delta^{15}N_{SourceAA}$ within primary producers, are far less variable in space and time than the bulk primary producer $\delta^{15}N$ values; and (3) the large TDFs for trophic AAs relative to bulk tissues provide greater TP separation power. Nevertheless, that the $\delta^{15}N_{baseline}$ is internally indexed does not negate the need to understand and potentially sample the broad taxonomic composition of primary producers supporting focal food webs, whose AA $\delta^{15}N$ values are explicitly required to parametrize the TP_{CSIA} equation (Eq. 1). These data are often assumed, yet not routinely collected (Figure 1), likely due to the perceived stability of these parameters based on earlier work and the fact that they are difficult measurements to make in comparison to analysis of protein-rich consumer tissues. However, we demonstrate that a large amount of variation is not currently reflected within these conventionally applied β values. Here, we outline four key recommendations for identifying, constraining, and accounting for β value variability that will improve characterization of consumer trophic status in

future TP_{CSIA} applications, particularly within ecosystems with complex primary producer assemblages.

(1) *Whenever feasible, sample the diverse suite of primary producers supporting focal food webs, particularly if they include vascular autotrophs.* All else being equal, *in-situ* sampling of primary producers concurrent to sampling of consumers remains the best method for identifying and constraining β values and TP_{CSIA} uncertainty. This will be particularly useful when studying species that forage within or across terrestrial, freshwater, and aquatic ecosystems, where β values may span 18‰ and are therefore likely to have the greatest influence on TP_{CSIA} estimates (e.g., Jarman et al., 2017; Figure 5). Such efforts will also help fill data gaps for the myriad of under-analyzed primary producer taxonomic groups (e.g., bryophytes, pteridophytes, and gymnosperms) and expand our understanding of ecologically relevant sources of β value variability (e.g., taxonomic, tissue type, mode of photosynthesis, N assimilation pathway). Subsequent recommendations pertain to scenarios where *in-situ* sampling is infeasible (e.g., historical ecology, archaeology).

(2) *Within systems dominated by vascular or non-vascular primary producers, use the most up to date β values and variance estimates, currently resulting from this meta-analysis, as starting points for TP_{CSIA} estimation (vascular = –6.6 ± 3.4‰; non-vascular = +3.3 ± 1.8‰; Tables 2, S1).* The β values in this meta-analysis integrate all published AA $\delta^{15}N$ data to date and thus most accurately reflect known β value variation among primary producers. Importantly, this recommendation includes propagation of the higher β value error estimates presented here using Monte Carlo simulation or simple Taylor expansion of the canonical TP_{CSIA} equation (Eq. 1) to estimate TP_{CSIA} uncertainty more accurately (e.g., through the *propagate* package in R; Gelwicks & Hayes, 1990). However, we encourage all CSIA-AA practitioners to carefully consider what β values would be most appropriate for their study system, including calculation of different β values using the raw data collated in this meta-analysis in combination with newly collected or published data in the future. Neverthelessour non-vascular β values may be appropriate to use in most marine applications where seagrasses are absent and terrestrial inputs are negligible (i.e., no vascular autotroph contribution) because known variability is relatively well-constrained. Within terrestrial systems, we encourage practitioners to further

constrain β values through purposeful identification of likely primary producer taxonomic groups and producer tissues that are important to focal food webs (e.g., grasses only, cacti only, estimated mix of woody and herbaceous plants).

(3) *For systems with both vascular and non-vascular primary producers, estimate β$_{mix}$ values using pertinent indices as endmembers in mixing models.* When consumers use food webs with both vascular and non-vascular autotrophs, β values must reflect the realized admixture of primary producers (i.e., β$_{mix}$) for each consumer (Choi et al., 2017; Ishikawa et al., 2014). Multiple molecular tools are available for use in tandem with mixing models to estimate the relative contribution of vascular and non-vascular primary producers to consumer food webs, such as bulk tissue $\delta^{13}C$ and $\delta^{15}N$ values (e.g., Choi et al., 2017; Zhang et al., 2019, 2021), AA $\delta^{13}C$ 'fingerprinting' (e.g., Bowes et al., 2020; Chua et al., 2020), fatty acid analysis (Hebert et al. 2016), and $\Delta_{Met\text{-}Phe}$ ($\delta^{15}N_{Met} - \delta^{15}N_{Phe}$; Ishikawa et al., 2018). Notably, phylogenetically distinct AA $\delta^{13}C$ 'fingerprints' can be used to differentiate resource use among a diverse array of baseline energy sources (e.g., terrestrial plants, algae, seagrasses, bacteria, fungi; Larsen et al. 2013). When combined with consumer AA $\delta^{15}N$ analyses, this collection of approaches can more accurately characterize consumer trophic dynamics in modern and historical food webs (e.g., Jarman et al., 2017).

(4) *Within systems dominated by vascular autotrophs, such as terrestrial food webs, consider using Lys instead of Phe as the source AA for TP$_{CSIA}$ estimation.* Our meta-analysis revealed that vascular plant β values calculated using the source AA Lys are considerably less variable across primary producer taxonomic groups and tissue types than those that use the source AA Phe (Figure 3), which may ultimately yield more precise TP$_{CSIA}$ estimates for food webs primarily supported by vascular autotrophs. The use of vascular β$_{Glx\text{-}Lys}$ is also advantageous because the data are approximately normally distributed and thus avoid distributional biases present in the vascular β$_{Glx\text{-}Phe}$ dataset (Figure 3). As a result, we advance the call for optimization of analytical protocols to collect the full suite of measurable AAs rather than just focusing on Glx and Phe (Bradley et al., 2015; Nielsen et al., 2015; McMahon & McCarthy, 2016; O'Connell, 2017). This recommendation comes with the caveat that Lys is sometimes more analytically challenging to measure on standard gas chromatography-combustion-isotope

ratio mass spectrometers given its late retention time and potential to co-elute with tyrosine (Tyr). With careful attention to late run temperature ramp structure, high quality Lys $\delta^{15}N$ data are achievable and likely worth the investment, particularly for terrestrial food web studies.

Conflicts of Interest

The authors declare that there is no conflict of interest.

Authors' Contributions

MDR, KWM, SDN, and ACB conceived the ideas and designed the methodology; MDR collected and analyzed the data; MDR and ACB led the writing of the manuscript; All authors contributed critically to the drafts and gave final approval for publication.

Data Availability Statement

Data and code available from Github (https://github.com/matthewdramirez/BetaValueMeta-analysis) and Zenodo (Ramirez et al. 2021).

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TABLE 1 Summary of published research devoted to characterizing amino acid $\delta^{15}N$ values in photosynthetic organisms (plants/macrophytes, eukaryotic macro- and microalgae, cyanobacteria, chemoautotrophic bacteria). See Figure 3 for characterization of tissue types analyzed. Dashes denote where genus-level identification was not reported.

Taxonomic group (n studies)			Sample Count by Cultivation Type	
	n_{genera}	$n_{samples}$	Natural	Culture
Marine (25)				
Eukaryotic microalgae	13	29	2	27
Ice algae	3+	7	7	0
Cyanobacteria	11	19	0	19
Macroalgae	10	16	15	1
Chemoautotrophic bacteria	3	18	0	18
POM	–	33	33	0
Seagrass	2	6	6	0
Freshwater (7)				
Eukaryotic microalgae	5+	41	41	0
POM	–	12	12	0
Macrophyte	8	17	17	0
Terrestrial (20)				
Moss	1	1	1	0
Cactus	2	5	5	0
Fern	1	1	1	0
Forb	22	72	15	57
Grass	17	100	14	86
Vine	4	8	4	4

Shrub	5	11	9	2
Tree	25	82	54	28
Leaf litter	–	36	36	0
Total (52)	132+	514	272	242

Note: Natural includes non-directly human manipulated/cultivated samples (e.g., wild plants). *Culture* includes directly human manipulated/cultivated samples, either in lab or farm settings. POM = particulate organic matter. n_{genera} is the number of autotroph genera analyzed; species-level identification was not always reported. $n_{samples}$ is the total number of autotrophs samples.

TABLE 2 $\beta_{Glx\text{-}Phe}$ and $\beta_{Glx\text{-}Lys}$ values (samples size, mean, standard deviation) by broad taxonomic groupings for data presented in Figures 3. See Table S1 for the full suite of summary $\beta_{X\text{-}Phe}$ and $\beta_{X\text{-}Lys}$ data and complementary $\beta_{X\text{-}Tyr}$ and $\beta_{X\text{-}Met}$ data.

Taxonomic group	$\beta_{Glx–Phe}$			$\beta_{Glx\text{-}Lys}$		
	n	Mean	SD	*n*	Mean	SD
Non-vascular Autotrophs[a]	68	3.3	1.8	20	4.2	2.9
Marine						
Eukaryotic algae (micro, macro, ice)	39	3.8	1.3	8	4.6	2.6
Cyanobacteria	19	2.1	2.2	8	4.3	3.4
Chemoautotroph	4	4.5	1.9	2	6.2	1.6
Freshwater						
Eukaryotic microalgae	5	4.2	0.7	1	1.1	—
Terrestrial						
Moss	1	0.1	—	1	0.6	—
Vascular Autotrophs[a]	152	–6.6	3.4	60	2.5	1.6
Marine						
Seagrass	2	–7.9	0.0	1	2.1	—
Freshwater						

Macrophyte	4	−6.9	1.8	1	−2.1	—
Terrestrial						
Cactus	3	−5.8	1.8	1	1.9	—
Fern	1	−6.0	—	1	4.7	—
Herbaceous (forb, grass)	66	−5.2	3.6	27	3.3	1.1
Woody (vine, shrub, tree)	76	−7.7	2.9	29	1.9	1.5

[a]Estimates exclude particulate organic matter and leaf litter.

TABLE 3 Consumer $\delta^{15}N_{Glx}$ and $\delta^{15}N_{Phe}$ data, baseline scenario parameters (β and trophic discrimination factors, $TDF_{Glx-Phe}$), and baseline scenario trophic position (TP_{CSIA}) estimates used to evaluate how variation in mean β values and TDFs influence mean TP_{CSIA} estimates (Figure 5). Max–Min TP_{CSIA} represents the difference between the maximum and minimum TP_{CSIA} estimated for each consumer within the sensitivity analysis.

Model systems and species	Consumer data			Baseline scenario			Max–Min TP_{CSIA}
	n	$\delta^{15}N_{Glx}$	$\delta^{15}N_{Phe}$	$\beta_{Glx-Phe}$	$TDF_{Glx-Phe}$	Baseline TP_{CSIA}	
(A) Terrestrial Food Chain							
Apple aphid[a] (*Aphis pomi*)	4	1.5	3.8	−6.50	7.5	1.6	1.85
Hoverfly[a] (*Eupeodes* sp.)	4	9.4	3.7	−6.50	7.5	2.6	2.05
Parasitoid wasp[a] (*Bothriothorax* sp.)	4	16.4	2.8	−6.50	7.5	3.7	2.34
Hyperparasitoid wasp[a] (*Pachyneuron albutius*)	4	24.0	3.2	−6.50	7.5	4.6	2.60
(B) Freshwater Food Chain							
Caddisfly larvae[b] (Hydropsychidae sp.)	3	10.9	0.0	−1.75	7.5	2.7	2.95
Trout[b] (*Oncorhynchus masou ishikawae*)	3	14.7	0.0	−1.75	7.5	3.6	4.08
Osprey[c] (*Pandion haliaetus*)	29	20.5	5.0	−1.75	4.5	3.5	5.14
(B) Oceanic Food Chain							
Copepod[d] (*Oithona* sp.)	4	10.0	−1.9	+3.25	7.5	2.2	1.14
Flying fish[e] (*Exocoetus volitans*)	1	15.0	−2.4	+3.25	7.5	2.9	1.06
Yellowfin tuna[e] (*Thunnus albacares*)	3	22.3	1.3	+3.25	5.5	3.9	1.85
Swordfish[e] (*Xiphias gladius*)	3	28.3	3.1	+3.25	5.5	4.6	2.13

[a]Steffan et al. (2013), [b]Ishikawa et al. (2014), [c]Elliott et al. (2021), [d]Hannides et al. (2009), [e]Choy et al. (2015)
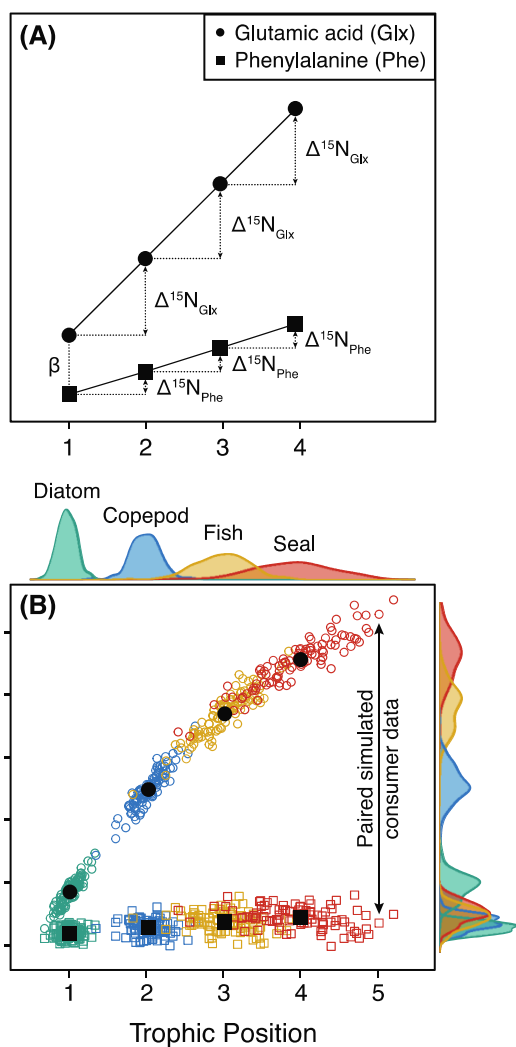
FIGURE 1 (A) Conceptual figure adapted from Chikaraishi et al. (2009) illustrating the simplified relationship between amino acid (AA) $\delta^{15}N$ values and trophic position (TP) within an aquatic system dominated by non-vascular primary producers. $\Delta^{15}N_{Glx}$ and $\Delta^{15}N_{Phe}$ are AA-specific trophic discrimination factors (TDFs) representing the change in trophic (e.g., Glx; circles, conventional mean ± SD = 8.0 ± 1.2‰) and source (e.g., Phe; squares, 0.4 ± 0.5‰) AA $\delta^{15}N$ value with each trophic transfer (TDF = $\Delta^{15}N_{Glx} - \Delta^{15}N_{Phe}$). β is the difference between trophic and source AA $\delta^{15}N$ values in the primary producer(s) at the base of the food web (+3.4 ± 0.9‰). **(B)** Simulation demonstrating how variation in β values and AA-specific TDFs propagate through a simple hypothetical food chain to influence consumer trophic position estimates (TP$_{CSIA}$). Within this simulation, $\Delta^{15}N_{Glx}$ declines from a mean of 8.0‰ to 6.1‰ to 4.4‰ with each trophic step and has a

SD of 1.2‰; $\Delta^{15}N_{Phe}$ has a mean ± SD of 0.4 ± 0.5‰ for all trophic transfers (McMahon & McCarthy 2016). For each model run, the initial offset between $\delta^{15}N_{Glx}$ and $\delta^{15}N_{Phe}$ in primary producer (TP_{CSIA} = 1) was defined by drawing random samples from both the conventional β value (+3.4 ± 0.9‰) and simulated Phe (+0.4 ± 0.5‰) distributions, which were then used to estimate Glx (i.e., estimated Glx = estimated Phe + estimated β). Glx and Phe estimates for the primary consumer (TP_{CSIA} = 2) were then generated by drawing random samples from the $\Delta^{15}N_{Glx}$ and $\Delta^{15}N_{Phe}$ distributions and adding them to the Glx and Phe estimates from the primary producer. This process was then repeated for the secondary (TP_{CSIA} = 3) and tertiary (TP_{CSIA} = 4) consumers. We ran the simulation 100 times, generating 100 hypothetical food chains. Once Glx and Phe estimates were generated for all model runs (i.e., 4 taxa * 100 simulations = 400 paired Glx and Phe estimates), we used these data in conjunction with the McMahon & McCarthy (2016) multi-TDF TP_{CSIA} equation to estimate TP_{CSIA} for each hypothetical consumer. For the TP_{CSIA} calculation we used a β of +3.4‰ and TDFs ($\Delta^{15}N_{Glx}$ − $\Delta^{15}N_{Phe}$) of 7.6‰ (primary consumer), 5.7‰ (secondary consumer), and 4.4‰ (tertiary consumer). Density plots illustrate the distributions of the simulated data, with TP_{CSIA} along the x-axis and Glx and Phe $\delta^{15}N$ values along the y-axis. Black circles and squares denote means.
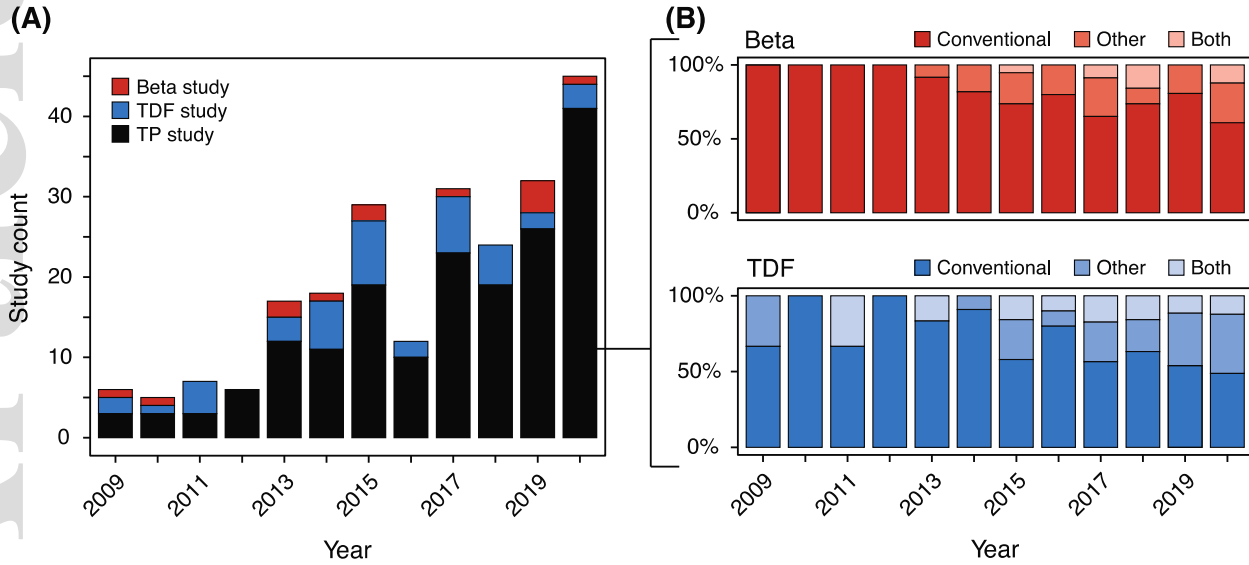
FIGURE 2 (**A**) Frequency distribution showing the growing number of published studies that use compound-specific stable isotope analysis of amino acids (CSIA-AA) to estimate consumer trophic position (TP_{CSIA}; black bars, Eq. 1, $n = 176$) and parameters of the TP_{CSIA} equation [Trophic discrimination factor (TDF), blue bars, $n = 44$; β, red bars, $n = 15$]. (**B**) Percent stacked bar charts illustrating the frequent use of conventional TDF and β values in TP_{CSIA} calculations, including the lagged consideration of non-conventional β values relative to non-conventional TDFs. *Conventional* indicates use of values from Chikaraishi et al. (2009, 2010; e.g., TDF = 7.6‰, β = +3.4 or −8.4‰); *Other* indicates use of non-conventional parameter values from other studies [e.g., Nielsen et al. (2015), Bradley et al. (2015), study-specific analysis, $β_{mix}$]; or *Both* (e.g., study uses a multi-TDF or multiple equations to calculate TP_{CSIA}).
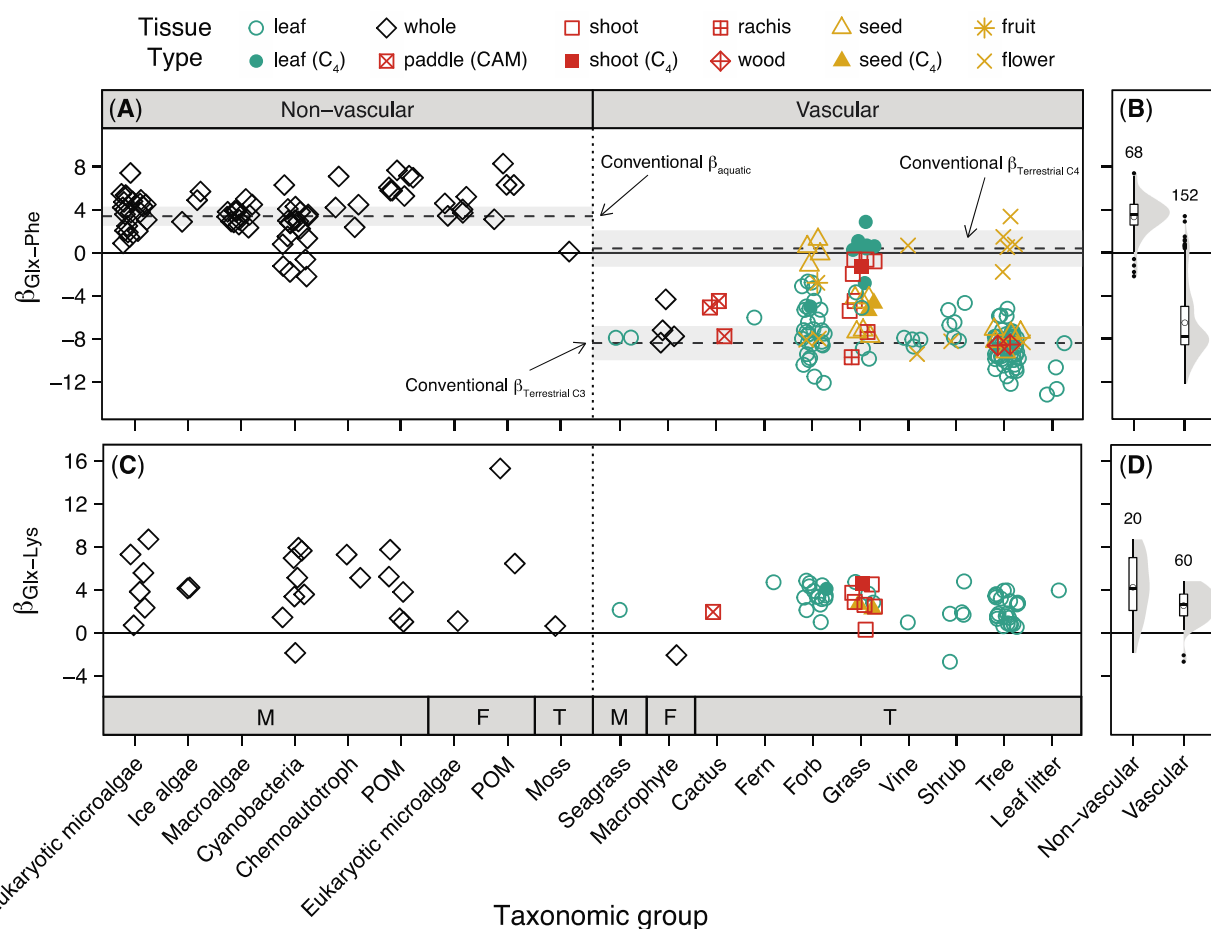
FIGURE 3 (**A, C**) $\beta_{Glx\text{-}Phe}$ and $\beta_{Glx\text{-}Lys}$ values collated from the published literature illustrating key sources of variability examined in this meta-analysis, including the presence/absence of a vascular system (subpanels), taxonomic group (jittered symbols), habitat type (M = marine, F = freshwater, T= terrestrial), tissue type (T only; colors, symbols), and mode of photosynthesis (T only; all C_3 unless otherwise noted). Dashed lines and shaded ribbons denote the conventionally applied β values (mean ± SD) for aquatic (3.4 ± 0.9‰; Chikaraishi et al. 2009), terrestrial C_3 (−8.4 ± 1.6‰; Chikaraishi et al. 2010), terrestrial C_4 (0.4 ± 1.7‰; Chikaraishi et al. 2010) primary producers. Particulate organic matter (POM) data are presented for comparison but were excluded from all analyses. (**B, D**) Half violin plots with inset boxplots showing the distribution of β values for vascular and non-vascular primary producers. Open circles denote groups means. Sample sizes are presented above each plot.
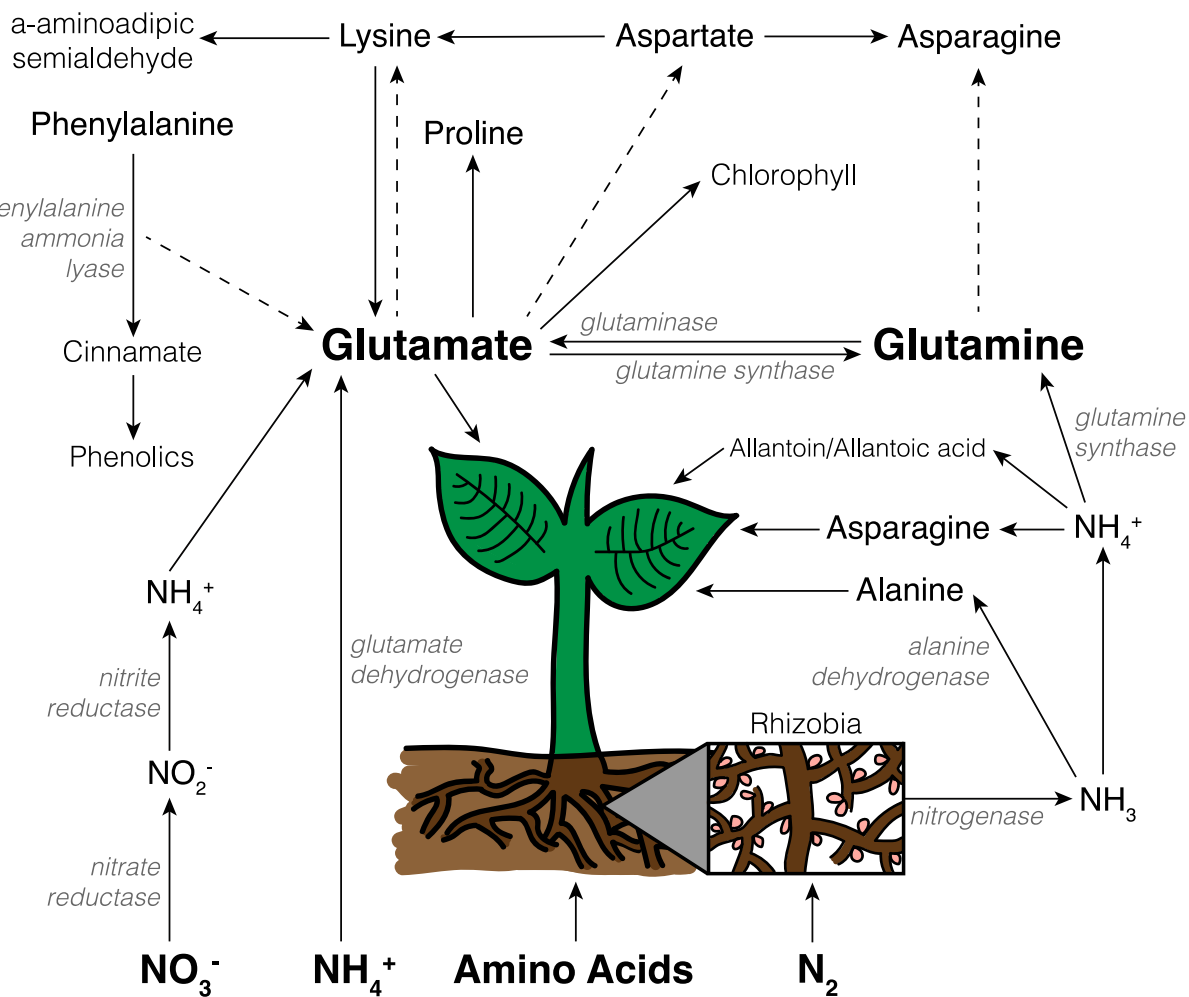
FIGURE 4 Schematic of primary producer nitrogen assimilation and metabolism. Inorganic (NO_3^-, NH_4^+, N_2) and organic [amino acids (AA)] nitrogen sources that can be acquired both directly by the plant and indirectly via symbiotic microbes are listed in bold at the bottom of the figure. Key pathways of transformation (enzymes in italics) and assimilation into plant AA are denoted by solid arrows. N_2 fixation occurs only in diazotrophs, including cyanobacteria and symbiotic rhizobia. Most aquatic and terrestrial primary producers are capable of assimilating nitrogen using any of the other pathways, though the extent of use varies by taxa and environmental conditions. Glutamate and Glutamine are at the center of nitrogen metabolism and serve as key nitrogen shuttles to other AA and metabolites. Dashed arrows indicate the movement of an amine group.
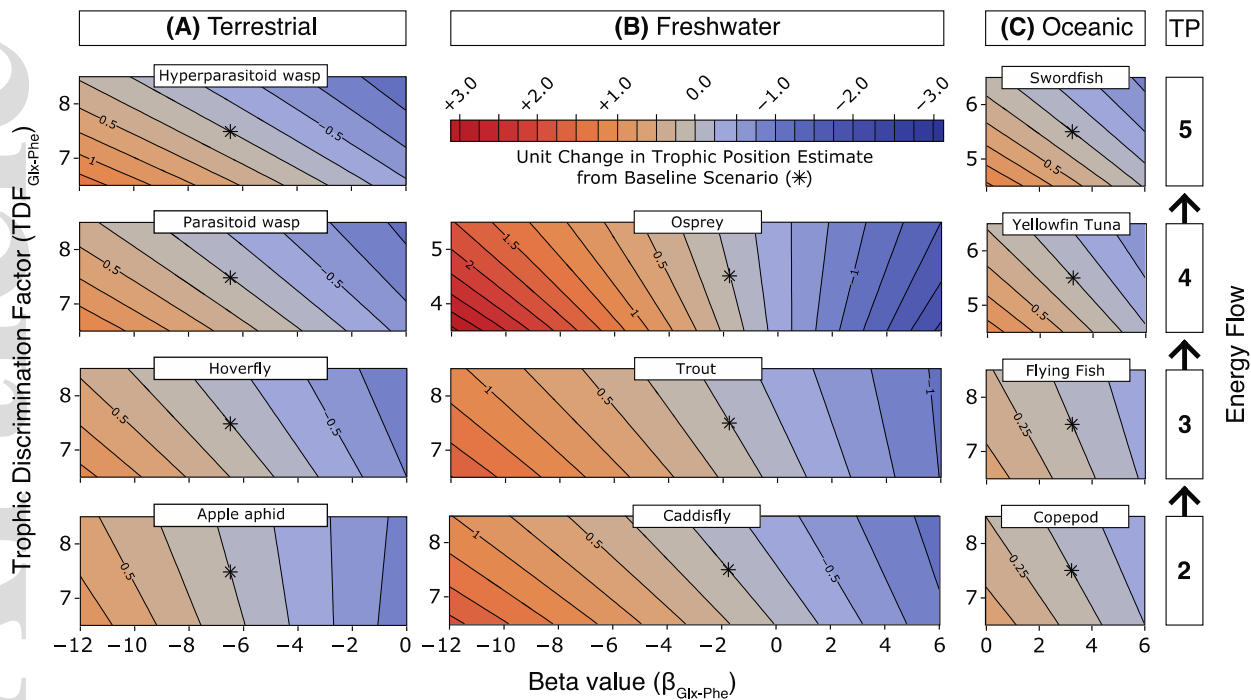
FIGURE 5 Change in consumer trophic position estimates (TP_{CSIA}) as a function of variation in mean beta values ($\beta_{Glx-Phe}$) and trophic discrimination factors ($TDF_{Glx-Phe}$) for 11 consumers within three model food chains: **(A)** terrestrial (vascular autotrophs only), **(B)** freshwater (both vascular and non-vascular autotrophs), and **(C)** oceanic (non-vascular autotrophs only). Plotted values reflect the difference between a consumer's TP_{CSIA} estimate *for a given β-TDF pairing* and the TP_{CSIA} estimate for the *baseline β-TDF pairing* scenario (stars). The baseline scenario was the TP_{CSIA} estimate derived from the mean TDF for a given panel (7.5 ‰ for primary and secondary consumers, 5.5 ‰ for 3°+ fish consumers, or 4.5 ‰ for 3°+ bird consumers) and a β value of –6.50 (**A**: terrestrial), –1.75 (**B**: freshwater), or +3.25 (**C**: oceanic) that approximate the mean vascular (–6.50) and non-vascular (+3.25) β values resulting from this meta-analysis or their mean (–1.75; i.e., assuming ~ 50% contribution of vascular and non-vascular autotrophs). Isoclines (diagonal black lines) bound bins reflective of a ± 0.25-unit change in trophic position from the baseline scenario TP_{CSIA} estimate. Underlying data were derived from the primary literature and used in conjunction with the single-TDF TP_{CSIA} equation (**A**, Chikaraishi et al., 2009) or multi-TDF TP_{CSIA} equation (**B, C**; McMahon & McCarthy, 2016) to estimate consumer TP_{CSIA}. Ranges of β values and TDFs followed known variation for each system and consumer type, such as reductions in mean TDFs with shifts in diet

quality or mode of nitrogen excretion (McMahon & McCarthy, 2016). See *Sensitivity of Trophic Position Estimates to Uncertainty in Beta Values* and Table 3 for list of data sources and justification for parameter means and ranges.