

## Embracing variability in amino acid $\delta^{15}\text{N}$ fractionation: mechanisms, implications, and applications for trophic ecology

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**Abstract.** Compound-specific stable isotope analysis (CSIA) of individual amino acids (AAs) has become a powerful analytical tool in trophic ecology. Heavily fractionating “trophic” AAs (e.g., glutamic acid: Glu) provide a robust indicator of trophic transfer, while minimally fractionating “source” AAs (e.g., phenylalanine: Phe) closely reflect the  $\delta^{15}\text{N}$  value at the base of the food web ( $\delta^{15}\text{N}_{\text{baseline}}$ ). Together, the CSIA-AA approach provides an unprecedented ability to disentangle the influences of  $\delta^{15}\text{N}_{\text{baseline}}$  values and trophic fractionation on consumer nitrogen isotope values. Perhaps the most important assumption underlying CSIA-AA applications to trophic ecology is that trophic fractionation of Glu and Phe, and thus the trophic discrimination factor  $\text{TDF}_{\text{Glu-Phe}}$  ( $\Delta^{15}\text{N}_{\text{Glu}} - \Delta^{15}\text{N}_{\text{Phe}}$ ), is effectively constant across diverse consumer–resource relationships. To test this assumption, we conducted a comprehensive meta-analysis of controlled feeding experiments that examined individual AA trophic fractionation ( $\Delta^{15}\text{N}_{\text{C-D}}$ ) and resulting  $\text{TDF}_{\text{Glu-Phe}}$  values. We found tremendous variability in  $\text{TDF}_{\text{Glu-Phe}}$  values from 0‰ to >10‰ across 70 species (317 individuals) and 88 distinct consumer–diet combinations. However, this variability appears to follow predictable patterns driven by two dominant variables: diet quality and mode of nitrogen excretion. Consumers feeding on high-quality diets (small diet–consumer AA imbalances) tend to have significantly lower  $\text{TDF}_{\text{Glu-Phe}}$  values than consumers feeding on low-quality diets. Similarly, urea/uric acid-producing consumers also exhibit significantly lower  $\text{TDF}_{\text{Glu-Phe}}$  values than their ammonia-producing counterparts. While these patterns are certainly not universal, together these factors likely explain many of the observed patterns of  $\text{TDF}_{\text{Glu-Phe}}$  variability. We provide an overview of the biochemical and physiological mechanisms underpinning AA  $\Delta^{15}\text{N}_{\text{C-D}}$  to explain these patterns. There are several seemingly unique systems, including the remarkably consistent  $\text{TDF}_{\text{Glu-Phe}}$  values across insect food webs and the isotopically “invisible” trophic transfers in microbial food webs, that may provide additional insight into the influence of diet quality and nitrogen cycling on AA fractionation. In this review, we argue that to realize the full potential of CSIA-AA approaches in trophic ecology, we must embrace the variability in  $\text{TDF}_{\text{Glu-Phe}}$  values. This likely requires developing new models of trophic transfer dynamics for some applications, including multi- $\text{TDF}_{\text{Glu-Phe}}$  equations that directly incorporate variability in  $\text{TDF}_{\text{Glu-Phe}}$  value.

**Key words:** amino acid; compound-specific stable isotope analysis; diet quality; food web; fractionation; nitrogen isotope; trophic discrimination factor; trophic ecology; trophic position.

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**EVOLUTION AND ASSUMPTIONS OF  
COMPOUND-SPECIFIC STABLE ISOTOPE ANALYSIS**

*Bulk stable isotopes in ecology: power and problems*

The concept of trophic position (TP) provides a valuable architectural framework for characterizing consumer–resource relationships within food webs (Lindeman 1942, Post 2002a). These interactions structure ecosystems via trophic cascades, mediate the relationship between species diversity and ecosystem function, and regulate both fisheries productivity and biogeochemical fluxes (Paine 1966, Carpenter et al. 1985, Cabana and Rasmussen 1994, Pace et al. 1999). As such, there is a rich history of literature seeking to develop and apply stable isotope analysis (SIA) to quantify resource utilization, trophic interactions, and the flow of energy through food webs (Vander Zanden and Rasmussen 1999, Post 2002b, Boecklen et al. 2011). Nitrogen stable isotope ratios ( $\delta^{15}\text{N}$ ) are particularly useful in determining trophic relationships. There is an old adage that says, “you are what you eat—plus some fractionation” (DeNiro and Epstein 1976), reflecting the fact that the  $\delta^{15}\text{N}$  value of a consumer reflects the weighted average  $\delta^{15}\text{N}$  values in its diet, with some alteration related to the biochemical and physiological transformation of the resources being utilized. This relationship is typically used to calculate a bulk trophic position ( $\text{TP}_{\text{Bulk}}$ ) based on the  $\delta^{15}\text{N}_{\text{baseline}}$  value at the base of the food web ( $\delta^{15}\text{N}_{\text{baseline}}$ ) and the fractionation of nitrogen between diet and consumer during trophic transfers

$$\Delta^{15}\text{N}_{\text{C-D}} = \delta^{15}\text{N}_{\text{consumer}} - \delta^{15}\text{N}_{\text{diet}} \quad (1)$$

The SIA approach has now become widely applied in trophic ecology (Boecklen et al. 2011), in part because it avoids many of the challenges inherent in the construction of food web networks using conventional gut content analysis and feeding observations (Deb 1997, Bearhop et al. 2004). However, there are a number of well-known challenges to interpreting bulk  $\delta^{15}\text{N}$  data, primarily linked to uncertainty in two key parameters: (1)  $\delta^{15}\text{N}_{\text{baseline}}$  and (2)  $\Delta^{15}\text{N}_{\text{C-D}}$ . First, many ecosystems are characterized by significant spatiotemporal variability in  $\delta^{15}\text{N}_{\text{baseline}}$  values (upwards of 10‰ in some places) owing to variations in the taxonomic

identity of primary producers at the base of the food web, the isotopically distinct sources of inorganic nitrogen ( $\text{N}_2$ , nitrate, ammonia) fueling those primary producers, and the efficiency with which nitrogen sources are utilized (McMahon et al. 2013a, b). Identifying appropriate  $\delta^{15}\text{N}_{\text{baseline}}$  values typically requires either extensive a priori characterization of the base of the food web or else broad assumptions about resource use (Vander Zanden and Rasmussen 1999, McCann et al. 2005). Second, the fractionation associated with trophic transfer ( $\Delta^{15}\text{N}_{\text{C-D}}$ ), often assumed to be 3.4‰, can vary widely (e.g., between -1‰ and 6‰) as a function of diet quality, tissue type, physiological stress, and biochemical form of nitrogenous waste (see reviews by Minagawa and Wada 1984, Vander Zanden and Rasmussen 2001, McCutchan et al. 2003). As a result, perhaps the central challenge to interpreting bulk tissue  $\delta^{15}\text{N}$  measurements, particularly of upper trophic-level consumers, is in determining whether consumer bulk  $\delta^{15}\text{N}$  values reflect variability in the  $\delta^{15}\text{N}_{\text{baseline}}$ , trophic positions,  $\Delta^{15}\text{N}_{\text{C-D}}$  values, or some combination of all of these factors (Post 2002b). Unfortunately, these intertwined drivers of consumer  $\delta^{15}\text{N}$  value simply cannot be resolved with bulk isotope measurements alone.

*Compound-specific stable isotope analysis of amino acid for trophic ecology*

Compound-specific stable isotope analysis (CSIA) of individual amino acids (AAs) has become a powerful analytical tool for ecologists, offering an unprecedented opportunity to disentangle the relative influences of  $\delta^{15}\text{N}_{\text{baseline}}$  and trophic fractionation on consumer  $\delta^{15}\text{N}$  value. The CSIA-AA approach has increased the accuracy and precision of trophic position estimates ( $\text{TP}_{\text{CSIA}}$ ) and provided a robust tracer of  $\delta^{15}\text{N}_{\text{baseline}}$ , all from a single sample of consumer tissue (e.g., McCarthy et al. 2007, Batista et al. 2014, Chikaraishi et al. 2014, Sherwood et al. 2014, Choy et al. 2015, Lorrain et al. 2015). Since the advent of continuous-flow gas chromatography–combustion–isotope ratio mass spectrometry (GC-C-IRMS) techniques, this approach has resulted in an explosion of CSIA-AA literature in recent years, crossing fields of ecology, biogeochemistry, archeology, and paleoceanography (Fig. 1).

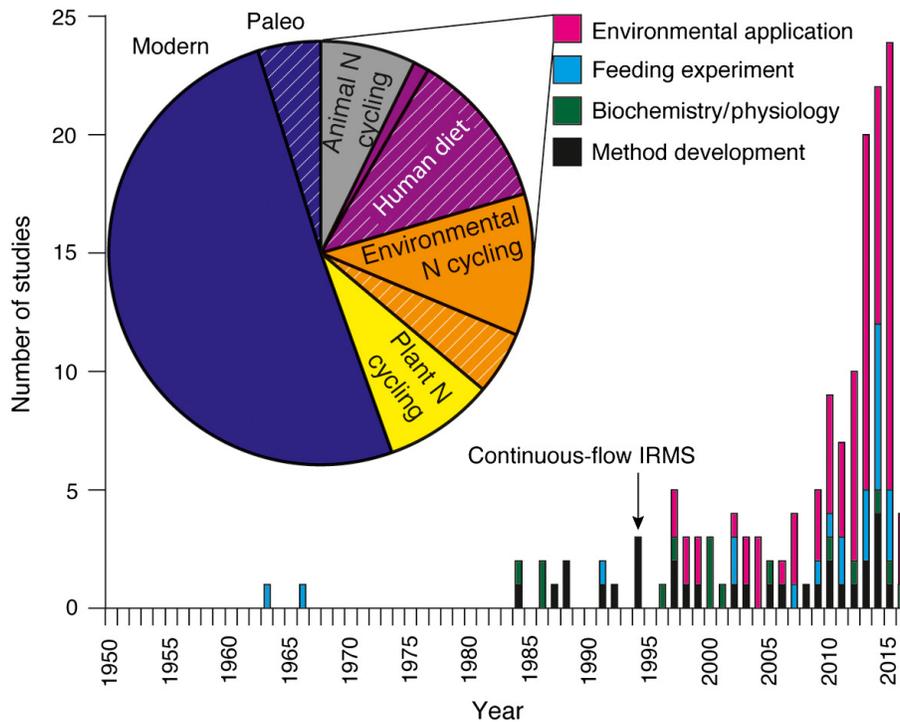


Fig. 1. A frequency distribution of published papers addressing compound-specific stable isotope analysis of individual amino acid (CSIA-AA) natural abundance  $\delta^{15}\text{N}$  values in the context of trophic ecology (arrow indicates advent of continuous-flow IRMS in 1994). We conducted a comprehensive search of Web of Science and Google Scholar using the key phrases “nitrogen isotope,” “amino acid,” and any of the following “trophic,” “diet,” and “food web.” The papers were sorted by subject area: (1) method development specific to CSIA-AA applied to trophic ecology (black bars), (2) biochemistry/physiology specific to fractionation of glutamic acid and phenylalanine (green bars), (3) controlled feeding experiments examining fractionation of individual amino acids between diet and consumer (cyan bars), and (4) environmental applications of CSIA-AA to trophic ecology (magenta bars). The inset shows the relative breakdown of the dominant environmental applications into the following subdisciplines: (1) trophic vs. baseline: calculating consumer trophic position relative to internally indexed food web baseline (blue), (2) animal N cycling: sources and cycling of nitrogen in animal consumers (gray), (3) human diet: protein sources for humans (purple), (4) environmental N cycling: sources and cycling of nitrogen in the environment (orange), (5) plant N cycling: sources and cycling of nitrogen within plants (yellow). Solid pie subsections represent modern applications and hatched pie subsections represent paleoapplications. References can be found in Appendix S2.

Differential  $^{15}\text{N}$  enrichment of individual AAs with trophic transfer is at the heart of the CSIA-AA approach to studying trophic ecology. Gaebler et al. (1963, 1966) were likely the first to document differential  $\delta^{15}\text{N}$  fractionation in AAs, showing that while some AAs exhibited large offsets in  $^{15}\text{N}$  excess in rat liver compared with diet, other AAs exhibited little or no isotope difference. Hare et al. (1991) also observed similar  $\delta^{15}\text{N}$  offsets in a smaller set of collagen AAs from pigs fed different diets. However, the implications of

these early observations for ecological research did not gain significant traction until the seminal paper by McClelland and Montoya (2002). This controlled feeding experiment on zooplankton was the first to explicitly divided protein AAs into two basic groups, based on the fractionation patterns of individual AAs, and put those patterns into an ecological context. McClelland and Montoya (2002) suggested that if appropriate calibrations could be developed, differential AA  $\delta^{15}\text{N}$  fractionation with trophic transfer could be

harnessed to yield independent information on both trophic level and  $\delta^{15}\text{N}_{\text{baseline}}$ . This is exactly what has happened in subsequent years.

In terms of  $\delta^{15}\text{N}$ , we now commonly divide protein AAs into two basic groups, termed the “trophic” and the “source” AAs (after Popp et al. 2007). The trophic AAs (glutamic acid: Glu; aspartic acid: Asp; alanine: Ala; isoleucine: Ile; leucine: Leu; proline: Pro; valine: Val) undergo significant isotopic fractionation (often  $\Delta^{15}\text{N}_{\text{C-D}} > 5\text{‰}$ ) during transamination and deamination, owing to their close linkage to the rapidly cycling internal glutamate pool (e.g., McCarthy et al. 2013). In contrast, the source AAs (phenylalanine: Phe; methionine: Met; lysine: Lys; tyrosine: Tyr) show relatively little trophic fractionation between  $\delta^{15}\text{N}_{\text{baseline}}$  and measured values in upper trophic-level consumers. While this division is sometimes confused with the more familiar essential vs. nonessential AA groupings for carbon, it is important to remember that these groupings not only represent different AAs (McMahon et al. 2013a), but are also based on fundamentally different basic biochemical mechanisms related to cycling of nitrogen vs. carbon-containing moieties of AA (e.g., McCarthy et al. 2013, McMahon et al. 2015c). The basic observations of generally consistent patterns in  $\delta^{15}\text{N}$  fractionation between trophic and source AAs have now been confirmed in multiple systems using multiple species (Chikaraishi et al. 2007, 2009, Germain et al. 2013, Steffan et al. 2013, McMahon et al. 2015a, b).

The ability to independently estimate the  $\delta^{15}\text{N}_{\text{baseline}}$  from source AAs and the number of trophic transfers from trophic AAs has now provided ecologists with a powerful tool to calculate consumer  $\text{TP}_{\text{CSIA}}$  that is internally indexed to the  $\delta^{15}\text{N}_{\text{baseline}}$ . The most common formulation for  $\text{TP}_{\text{CSIA}}$  is based on the offset between the canonical trophic AA Glu and source AA Phe:

$$\text{TP}_{\text{CSIA}} = 1 + \left[ \frac{\delta^{15}\text{N}_{\text{Glu}} - \delta^{15}\text{N}_{\text{Phe}} - \beta}{\text{TDF}_{\text{Glu-Phe}}} \right] \quad (2)$$

where  $\delta^{15}\text{N}_{\text{Glu}}$  and  $\delta^{15}\text{N}_{\text{Phe}}$  represent the stable nitrogen isotope values of the consumer Glu and Phe, respectively,  $\beta$  represents the difference in  $\delta^{15}\text{N}$  between these same AAs in primary producers at the base of the food web, and  $\text{TDF}_{\text{Glu-Phe}}$  represents the trophic discrimination

factor (TDF) between diet and consumer, calculated by normalizing trophic fractionation of Glu ( $\Delta^{15}\text{N}_{\text{Glu}}$ ) to Phe ( $\Delta^{15}\text{N}_{\text{Phe}}$ ),

$$\text{TDF}_{\text{Glu-Phe}} = \Delta^{15}\text{N}_{\text{Glu}} - \Delta^{15}\text{N}_{\text{Phe}}. \quad (3)$$

Perhaps the most important assumption underlying common CSIA-AA applications to trophic ecology is that trophic fractionation of Glu and Phe, and thus  $\text{TDF}_{\text{Glu-Phe}}$  is effectively constant across diverse consumer–resource relationships. Chikaraishi et al. (2007) originally proposed an average  $\text{TDF}_{\text{Glu-Phe}}$  value of 7.6‰ in the first study to undertake extensive feeding trials with multiple marine consumers. Their  $\text{TDF}_{\text{Glu-Phe}}$  results were in fact almost identical to the  $\text{TDF}_{\text{Glu-Phe}}$  value derived from McClelland and Montoya’s (2002) original rotifer feeding experiments (7.0‰). As a result, a “universal”  $\text{TDF}_{\text{Glu-Phe}}$  value of 7.6‰ became essentially a canonical value that has been adopted widely for calculating  $\text{TP}_{\text{CSIA}}$  across multiple taxa and environments (e.g., Lorrain et al. 2009, 2015, Dale et al. 2011, Choy et al. 2012, Miller et al. 2013, Nakatomi et al. 2014).

#### *Trouble in paradise: questioning the “universality” of 7.6‰*

In recent years, the basic assumption of a constant  $\text{TDF}_{\text{Glu-Phe}}$  value has come under increasing scrutiny. A number of field studies calculating  $\text{TP}_{\text{CSIA}}$  in upper trophic-level consumers (including cephalopods, teleost fishes, elasmobranchs, marine mammals, and penguins) have noted that assuming a constant  $\text{TDF}_{\text{Glu-Phe}}$  value of 7.6‰ often led to substantially underestimated  $\text{TP}_{\text{CSIA}}$  relative to expected values from a whole host of other metrics, including gut content analysis and feeding observations (Lorrain et al. 2009, Dale et al. 2011, Choy et al. 2012, Ruiz-Cooley et al. 2013, 2014, Matthews and Ferguson 2014). Bradley et al. (2015) used an elegant linear regression approach to back-calculate a  $\text{TDF}_{\text{Glu-Phe}}$  value of  $5.7\text{‰} \pm 0.3\text{‰}$  from 224 wild-caught teleost fishes (47 species) based on the difference in consumer  $\delta^{15}\text{N}_{\text{Glu}}$  and  $\delta^{15}\text{N}_{\text{Phe}}$  values and predicted TP from published stomach content analyses. Using a similar linear regression approach, Nielsen et al. (2015) found a mean  $\text{TDF}_{\text{Glu-Phe}}$  value of  $6.6\text{‰} \pm 1.7\text{‰}$  for a meta-analysis of 359 diverse marine consumers (including

invertebrates, fishes, marine mammals, marine reptiles, and marine birds). While these studies clearly showed that the  $TDF_{\text{Glu-Phe}}$  value is not always 7.6‰, at the same time, the inherent nature of approaches focused on a single average value belies the full complexity in  $TDF_{\text{Glu-Phe}}$  variability.

Around the same time, a number of controlled feeding experiments targeting upper trophic-level marine consumers that were conspicuously absent from the earlier laboratory investigations have subsequently showed that  $TDF_{\text{Glu-Phe}}$  values can vary by an order of magnitude across different consumer–resource relationships (e.g., Germain et al. 2013, Bradley et al. 2014, Hoen et al. 2014, Chikaraishi et al. 2015, McMahon et al. 2015a, b). Perhaps most important, however, is that such studies also strongly suggest that this variation is not “noise,” but rather it is mechanistically linked to variations in animal physiology and biochemistry. Therefore, we suggest that the central question for CSIA-AA applications in trophic ecology may no longer be “what is the right TDF value” but rather “will any single-TDF value approach be sufficient to realize the full potential of CSIA-AA for trophic ecology?”

#### *Goals of this review*

The accuracy of  $TP_{\text{CSIA}}$  estimates, and thus the applicability of CSIA-AA to trophic ecology, is critically dependent upon the accuracy of  $TDF_{\text{Glu-Phe}}$  values, which we now know can vary substantially across diverse consumer–resource relationships. We begin our review by examining the fundamental underpinnings of  $TDF_{\text{Glu-Phe}}$  variability, including a consideration of the magnitude and mechanisms of fractionation in the trophic and source AAs. We do this with a comprehensive meta-analysis of controlled feeding experiments that examine individual AA  $\Delta^{15}\text{N}$  (see Appendix S1 for methods on the meta-analysis). Next, we identify and discuss the most likely systematic drivers of this variation, summarizing evidence for the influences of diet quality and mode of nitrogen excretion on  $TDF_{\text{Glu-Phe}}$  value. We also examine two systems with rather unique patterns of AA  $\delta^{15}\text{N}$  fractionation (insect vs. noninsect systems and marine prokaryotic systems) to help refine our understanding of mechanisms of AA  $\delta^{15}\text{N}$  fractionation. Finally, we close with a call for the development and

testing of new CSIA-AA models for calculating  $TP_{\text{CSIA}}$ , in particular multi-TDF and multi-AA approaches, that explicitly incorporate our growing understanding of the systematic variability in individual AA fractionation.

#### **NITROGEN ISOTOPE FRACTIONATION IN AMINO ACIDS**

The fate of AAs during metabolism (reviewed in Wu 2009), be it building blocks for the biosynthesis of proteins, fuel for energy, precursors for other nitrogenous substances, or waste components for nitrogen homeostasis, plays a key role in the cycling and subsequent isotope fractionation of nitrogen in organisms. As such, a solid understanding of AA metabolism is crucial for CSIA-AA applications in nutritional and ecological studies (Fig. 2A). However, past reviews of CSIA-AA have rarely addressed the underlying biochemical mechanisms of AA isotope fractionation in any detailed or unified way. In this section, we therefore review the basics of AA metabolism as it relates to the cycling of nitrogen in organisms. We then describe patterns of individual AA nitrogen isotope fractionation during typical metabolic processes (see Supplement 1 in Data S1 for meta-analysis summary table of  $\Delta^{15}\text{N}_{\text{C-D}}$  values). Together, these aspects form the theoretical basis for the subsequent sections addressing the application of individual AA nitrogen isotope fractionation patterns to trophic ecology.

#### *Transamination and deamination of amino acids*

Nitrogen for AA biosynthesis comes from several distinct sources, including exogenous supply from the diet, endogenous supply from the body nitrogen pool, and in some cases symbiotic supply from gut microbial communities (Felig 1975, Bender 2012, Ayayee et al. 2014). The enzymatic actions of AA metabolic pathways link these nitrogen sources to their ultimate destination. As such, isotopic discrimination of AA nitrogen is dependent upon the number and isotope effect of enzymatic reactions, as well as the flux of nitrogen through these pathways (Handley and Raven 1992, Webb et al. 1998, Ohkouchi et al. 2015).

Transamination and deamination are the two dominant enzymatic processes that control the

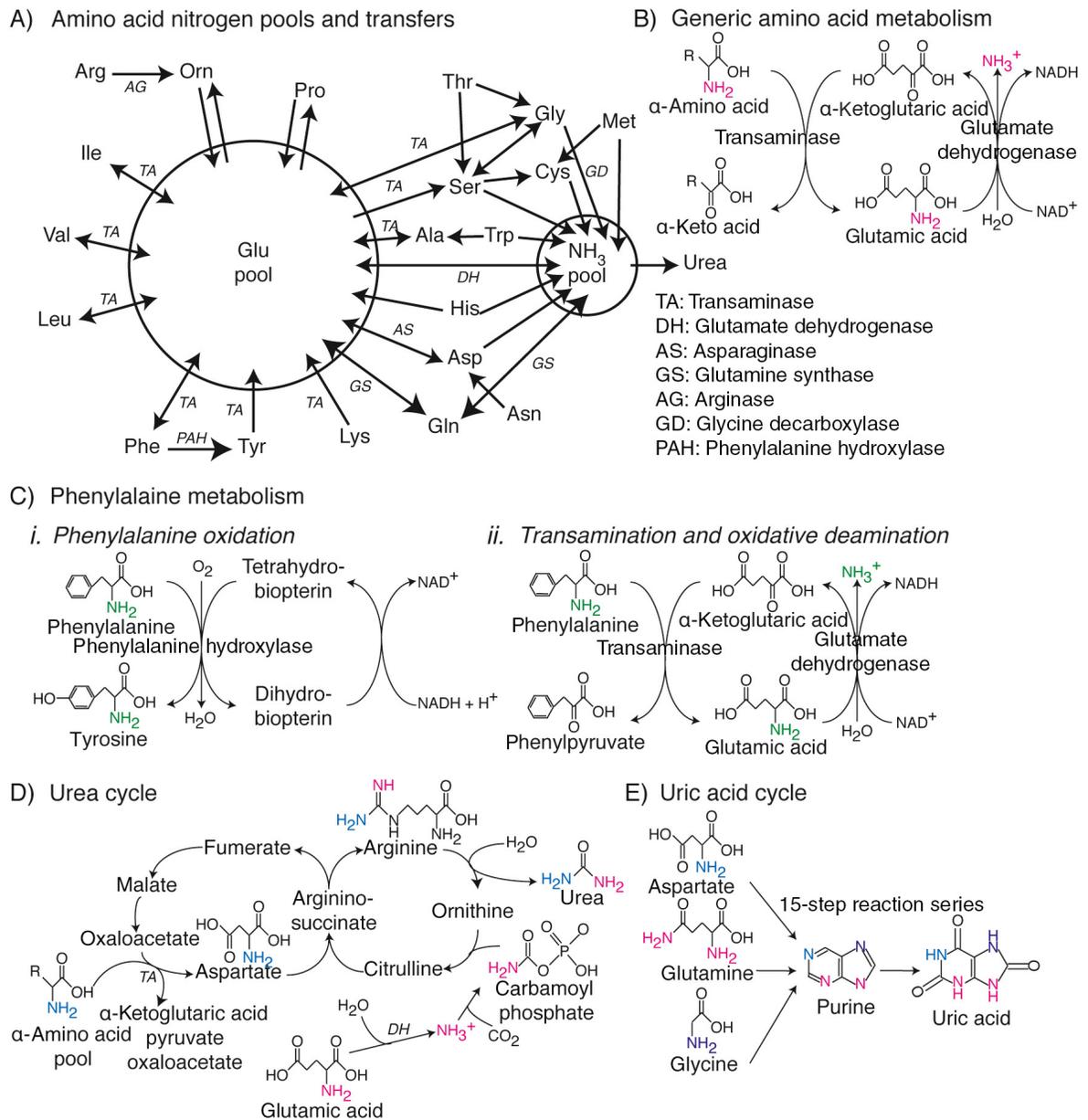


Fig. 2. (A) Typical nitrogen pools and associated transfers for individual amino acids (in standard three-letter abbreviations) during animal metabolism (modified from Braun et al. 2014). Double arrows reflect reversible reactions, while single arrows reflect irreversible or multistep reactions. Key enzymatic reactions are indicated in italics next to the arrows. (B) General amino acid metabolism through the transamination and oxidative deamination pathways linked to glutamic acid. (C) Phenylalanine metabolism through (i) the dominant pathway of conversion to tyrosine via phenylalanine hydroxylase without significant nitrogen isotope fractionation and (ii) transamination and oxidative deamination to phenylpyruvate with significant nitrogen isotope fractionation. (D) Simplified urea cycle through the aspartate–argininosuccinate shunt of the citric acid cycle. The nitrogen in cyan and magenta was transferred to urea via aspartate and carbamoyl phosphate (ultimately from glutamic acid), respectively. (E) One component of the simplified uric acid cycle showing sources of nitrogen to uric acid via purine catabolism. The nitrogen in cyan, magenta, and dark blue was transferred to uric acid via aspartate, glutamine, and glycine, respectively, through a series of 15 enzymatic reactions.

flow of nitrogen, and thus nitrogen isotope fractionation, in proteinaceous AAs. Transamination refers to the transfer of an amine group on one ketone-containing acid (e.g., AA) to another (e.g., keto acid) in a reaction catalyzed by a family of enzymes called transaminases or aminotransferases (Fig. 2B). Given that most transamination reactions have equilibrium constants near 1 (Handley and Raven 1992), the direction of transamination reactions is largely dictated by the relative intracellular concentrations of the reactants (Mathews et al. 2012). Deamination refers to the removal of an amine group from a molecule via deaminase enzymes and is the process by which AAs are broken down to liberate ammonia (Fig. 2B). Glutamate dehydrogenase is the dominant enzyme involved in the oxidative deamination of Glu to  $\alpha$ -ketoglutaric acid and ammonia, while dehydratase is primarily responsible for the nonoxidative deamination of AAs containing hydroxyl group, such as serine (Ser) and threonine (Thr). The liberated ammonia from deamination can be used for other biosynthetic pathways or excreted as nitrogenous waste.

Both transamination and deamination, like nearly all enzyme-mediated reactions, favor the lighter stable isotope ( $^{14}\text{N}$ -containing amine groups; Macko et al. 1986) via kinetic fractionation. Given the diversity of transaminases acting upon AAs, each with different isotope effects, coupled with the fact that AAs can also differ in the degree to which they are transaminated (Bowes and Thorp 2015), there is a wide potential range in the nitrogen isotope fractionation of individual AAs. In the subsequent subsections, we explore the patterns of fractionation for different AAs based upon their degree of transamination and deamination, with an emphasis on the implications for understanding CSIA-AA applications to trophic ecology.

#### *The heavily fractionating “trophic” amino acids*

The trophic AAs (e.g., Glu, Asp, Ala, Ile, Leu, Pro, Val) generally exhibit large positive increases in  $\delta^{15}\text{N}$  values with trophic transfer (Fig. 3). This is because all of these trophic AAs either undergo extensive transamination/deamination reactions associated with Glu and the central nitrogen pool or are directly linked to AAs that do (Fig. 2A). Glu, which is typically one of the more abundant AAs in consumer tissues, is often considered the

canonical trophic AA. In our meta-analysis, Glu had the highest mean  $\Delta^{15}\text{N}_{\text{C-D}}$  value of all AAs ( $\Delta^{15}\text{N}_{\text{C-D}} = 6.4\text{‰} \pm 2.5\text{‰}$ ). The substantial fractionation of Glu during trophic transfer is due to extensive transamination and deamination during metabolic processing, which leaves the residual Glu pool  $^{15}\text{N}$ -enriched (Fig. 2B). It is important to note that acid hydrolysis converts glutamine (Gln) and asparagine (Asn) into Glu and Asp, respectively, resulting in the measurement of combined Gln + Glu (referred to hereby as Glu) and Asn + Asp (referred to hereby as Asp). While some researchers referred to these groupings as Glx and Asx, we chose our terminology here to be consistent with most other CSIA studies.

The remaining trophic AAs typically exhibit fractionation patterns that closely resemble those of Glu (Fig. 3). This is because transamination reactions are often chained together to provide a continuous redistribution and homogenization of nitrogen among transaminating AAs linked to the central nitrogen pool via Glu (Fig. 2A; Nakada 1964, Kalhan and Parimi 2006, Mathews et al. 2012). While understanding the underlying biochemical transformations of individual AAs will help predict their fractionation patterns, there is still some uncertainty remaining in the magnitude of specific fractionations during metabolic processing. This is true for all AAs, even those typically referred to as “source” below. Future work linking the flux of AAs through biochemical pathways and the associated isotope effects of those pathways will greatly improve our understanding of AA fractionation during trophic transfer.

*Glycine and serine: troubles in classification.*— Glycine (Gly) and Ser are two notoriously challenging AAs to classify into the conventional “trophic” and “source” framework. These AAs were both originally termed “source” AAs (Popp et al. 2007) based largely on the results of the original McClelland and Montoya (2002) study that found minimal trophic fractionation for Gly (mean =  $0.9\text{‰} \pm 0.9\text{‰}$ ) and Ser (mean =  $0.8\text{‰} \pm 0.1\text{‰}$ ) between marine rotifers and their microalgal diet. More recent evidence suggests that in marine planktonic food webs in general, these AAs in fact may have relatively low  $\Delta^{15}\text{N}_{\text{C-D}}$  values (McCarthy et al. 2007; Mompean et al., 2016). However, across the broader range of

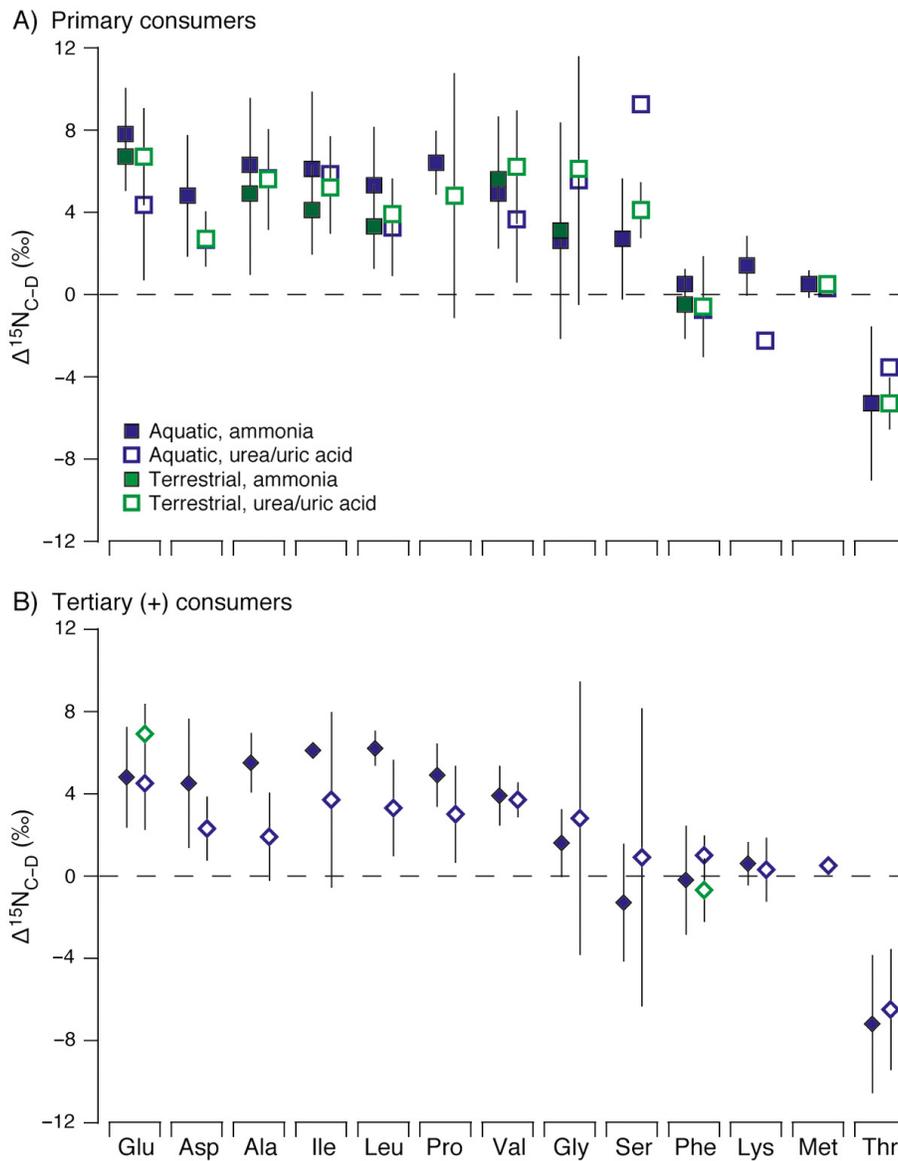


Fig. 3. Mean individual amino acid ( $\% \pm SD$ ) trophic fractionation factors ( $\Delta^{15}N_{C-D} = \delta^{15}N_{\text{consumer}} - \delta^{15}N_{\text{diet}}$ ) for (A) primary consumers (squares) and (B) tertiary (and higher) consumers (diamonds) in controlled feeding studies and well-constrained field collections: aquatic: marine and freshwater (blue symbols) vs. terrestrial (green symbols) and ammonia producers (closed symbols) vs. urea/uric acid producers (open symbols).

consumers in our meta-analysis, the variability in  $\Delta^{15}N_{C-D}$  values is extremely large for both Gly (mean =  $3.9\% \pm 4.9\%$ , max =  $14.2\%$ , min =  $-6.9\%$ ) and Ser (mean =  $2.9\% \pm 4.6\%$ , max =  $9.7\%$ , min =  $-5.8\%$ ; Fig. 3). This is likely because Gly and Ser can be readily linked both to the heavily transaminating central nitrogen pool via Glu and to ammonia and uric acid production (Fig. 2A;

Matthews et al. 1981, Hoskin et al. 2001). Finally, Gly is also strongly affected by microbial degradation, in terms of both its concentration and its  $\delta^{15}N$  values (e.g., McCarthy et al. 2007, Calleja et al. 2013). This would suggest additional caution must be taken in using Gly as a source AA in any sample types where microbial degradation or direct microbial contribution is

important. Given the large and highly variability  $\Delta^{15}\text{N}_{\text{C-D}}$  values of these transaminating, non-essential AAs, we suggest that the use of Gly and Ser as “source” AAs should be treated with great caution, in particular in any nonplankton consumers. Nielsen et al. (2015) recently reached a similar conclusion based on their meta-analysis of wild-caught marine consumers.

*The peculiar case of threonine.*—As with Gly and Ser, Thr was originally classified as a “source” AA based largely on the early study by McClelland and Montoya (2002). However, our meta-analysis (mean Thr  $\Delta^{15}\text{N}_{\text{C-D}} = -5.8 \pm 3.2$ ) provides further support to a growing body of literature, indicating that Thr nitrogen isotope fractionation behaves completely differently than any other AA, routinely exhibiting significant depletion during trophic transfer. Thr does not undergo reversible transamination reactions (Hoskin et al. 2001, Bergen and Wu 2009, Braun et al. 2014); however, an explanation for this peculiar fractionation pattern is not yet clear. Hare et al. (1991) suggested that Thr catabolism may result in an unusual inverse isotope effect, whereby the enzyme selects for the heavy isotope, leaving the residual Thr  $^{15}\text{N}$ -depleted. These authors hypothesized that Thr  $\delta^{15}\text{N}$  may constitute a marker of dietary stress. Others have noted strong negative relationships between Thr and TP, suggesting a link between Thr nitrogen isotope fractionation and trophic transfer (Bradley et al. 2015; Mompean et al. 2016). McMahon et al. (2015a) suggested that the degree of nitrogen isotope fractionation in Thr may also be directly related to diet quality, similar to the trophic AAs (discussed in *Variability in Trophic...: Diet quality: the master variable?*). Given the unique “inverse” fractionation with trophic transfer, recent papers have begun to classify Thr into its own category, sometimes termed a “metabolic” AA (e.g., Germain et al. 2013, McCarthy et al. 2013, Batista et al. 2014, McMahon et al. 2015a, b).

#### *The minimally fractionating “source” amino acids*

While most AAs undergo substantial fractionation during transamination/deamination processes linked to the central Glu nitrogen pool, there are a few AAs that appear to show minimal fractionation during trophic transfer. The  $\delta^{15}\text{N}$  values of these “source” AAs are therefore

thought to directly reflect  $\delta^{15}\text{N}_{\text{baseline}}$  values without the confounding issue of trophic fractionation. As such, the one of the major advantages of the CSIA-AA approach in trophic ecology is that it does not require a priori characterization of the baseline or detailed knowledge of all trophic connections in order to link upper trophic-level consumers to  $\delta^{15}\text{N}_{\text{baseline}}$  values. This is particularly valuable when working in complex or dynamic systems, where multiple different baseline end-members are present (e.g., Ishikawa et al. 2014, Maki et al. 2014, Ruiz-Cooley et al. 2014), when working on highly mobile or high-trophic-level consumers that may be integrating across multiple food webs (e.g., Lorrain et al. 2009, Dale et al. 2011, Papastamatiou et al. 2015), or perhaps most strikingly, in paleoapplications where we generally lack preservation of baseline end-members completely (e.g., Itahashi et al. 2014, Sherwood et al. 2014, Schwartz-Narbonne et al. 2015).

As noted above, a number of AAs have been variously designated as “source” AAs, including Phe, Met, Tyr, Gly, Ser, Thr, and Lys (McCarthy et al. 2007, Popp et al. 2007, Bradley et al. 2015, Nielsen et al. 2015), largely based on early feeding studies (McClelland and Montoya 2002, Chikaraishi et al. 2007). However, many of these AAs have since been shown to undergo substantial change in  $\delta^{15}\text{N}$  with trophic transfer (e.g., Gly, Ser, Thr). Understanding the underlying biochemical mechanisms of AA nitrogen isotope fractionation may be the only way to accurately assess the relative stability of these AAs across diverse consumer–resource relationships.

*The canonical source amino acid, phenylalanine.*—Phe is typically considered the canonical “source” AA. In our meta-analysis, Phe indeed showed the lowest trophic fractionation values ( $\Delta^{15}\text{N}_{\text{C-D}} = -0.1\text{‰} \pm 1.6\text{‰}$ ) across a diverse suite of consumer–resource relationships (Fig. 3). The dominant metabolic pathway for metabolism of excess dietary Phe is hydroxylation to Tyr by the enzyme phenylalanine hydroxylase (Fig. 2Ci). This process does not form or break C–N bonds and thus does not impart nitrogen isotope fractionation. As such, a number of studies have used  $\delta^{15}\text{N}_{\text{Phe}}$  values to calculate  $\delta^{15}\text{N}_{\text{baseline}}$  (e.g., Lorrain et al. 2009, 2015, Sherwood et al. 2014, Vokhshoori and McCarthy

2014). However, as the substantial standard deviation around this mean ( $\pm 1.6\%$ ) makes clear, Phe can and does undergo significant fractionation in some cases. A number of studies working with isotopically labeled  $^{15}\text{N}$  tracers have directly shown that dietary nitrogen does become incorporated into Phe, albeit at low levels relative to most other AAs (Gaebler et al. 1966, Hoskin et al. 2001).

The question becomes, why does Phe typically show minimal trophic fractionation, and perhaps more importantly, under what conditions does fractionation of Phe  $\delta^{15}\text{N}$  values become significant? The answer likely lies in the relative importance of metabolic pathways for Phe (Chikaraishi et al. 2007, 2009). In addition to the nonfractionating metabolic pathway for Phe (Fig. 2Ci), a second metabolic pathway exists where Phe is transaminated to phenylpyruvate (Fig. 2Cii). As with all transamination reactions, this process involves breaking C–N bonds of the amine group and thus does impart isotope fractionation. In most healthy organisms, the transamination pathway for Phe is relatively minor, imparting only a small fractionation during trophic transfer and metabolic processing (but see examples where the hydroxylase pathway for Phe metabolism is blocked, e.g., phenylketonuria [Blau et al. 2010]).

Overall, the typically small trophic fractionation of Phe may not pose serious issues when determining  $\delta^{15}\text{N}_{\text{baseline}}$  values in low-trophic-level consumers with relatively few trophic transfers from the baseline. However, when dealing with high-trophic-level consumers, even a small Phe  $\Delta^{15}\text{N}_{\text{C-D}}$  value (e.g.,  $0.7\%$  Chikaraishi et al. 2009), if propagated through four or more trophic transfers, would impart a significant shift in consumer  $\delta^{15}\text{N}_{\text{Phe}}$  value relative to the  $\delta^{15}\text{N}_{\text{baseline}}$  value. For example, when trying to estimate accurate  $\delta^{15}\text{N}_{\text{baseline}}$  values from sperm whales (*Physeter macrocephalus*, TP > 4), Ruiz-Cooley et al. (2014) had to apply a significant correction to the  $\delta^{15}\text{N}_{\text{Phe}}$  values of these apex predators to account for the propagation of Phe  $\Delta^{15}\text{N}_{\text{C-D}}$  across four trophic transfers.

*Other useful "source" amino acids: methionine and lysine.*—Similar to Phe, Met is also a potentially valuable source AA for recording  $\delta^{15}\text{N}_{\text{baseline}}$  (Chikaraishi et al. 2007). While there is the potential for transamination of Met

via methionine adenosyltransferase (Case 1976, Blom et al. 1989), the primary metabolic pathway of Met involves transsulfuration to other sulfur-containing AAs without forming or breaking C–N bonds and thus without significant isotopic fractionation (Stipanuk 1986). As a result, Met had a relatively small and consistent  $\Delta^{15}\text{N}_{\text{C-D}}$  value in our meta-analysis ( $0.4\% \pm 0.4\%$ ). However, in practical terms, the generally low abundance of Met in animal tissues (Beach et al. 1943) means that Met may not always be amenable to routine CSIA-AA applications. Only seven of the 88 species–diet combinations in our meta-analysis reported Met  $\Delta^{15}\text{N}_{\text{C-D}}$  values.

Lys is another AA that is commonly included within the source AA category and typically has the highest molar percent abundance of the source AAs (Beach et al. 1943). Lys metabolism is a bit unusual because it contains two nitrogen groups, including an amino group at the end of a four-carbon aliphatic side chain. There are at least three pathways for Lys catabolism, but the primary pathway (in mammals) results in the irreversible transamination of Lys to saccharopine and then Glu, which are subsequently subjected to deamination and oxidation (Tomé and Bos 2007). As such, the  $\delta^{15}\text{N}$  value of Lys is not homogenized with the rest of the central nitrogen pool of transaminating AAs. The generally low  $\Delta^{15}\text{N}_{\text{C-D}}$  of Lys in our meta-analysis ( $0.8\% \pm 1.5\%$ ) supports this assertion.

*Consideration of gut microbe contributions of source amino acids.*—A final, but important, consideration for using source AAs as proxies for  $\delta^{15}\text{N}_{\text{baseline}}$  is the assumption these AAs are derived only from diet and therefore reflect environmental primary production. De novo synthesized source AAs from gut microbes can be an important secondary supply of source AAs, particularly in organisms feeding on low protein diets (McBee 1971, Harris 1993, Clements et al. 2009, Newsome et al. 2011). While further research is needed to fully understand the conditions under which gut microbes contribute AAs to consumer tissues, the diversity of nitrogen sources available to gut microbes, coupled with their ability to de novo synthesize all AAs (Torrallardona et al. 1996, Metges 2000), presents a mechanism that can decouple consumer source AA  $\delta^{15}\text{N}$  values and

$\delta^{15}\text{N}_{\text{baseline}}$  values under some conditions (Harris 1993).

## VARIABILITY IN TROPHIC DISCRIMINATION FACTORS

The previous section discussed the basis of differential fractionation in individual AAs. However in practice, it is the trophic discrimination factor, typically defined as the difference in  $\Delta^{15}\text{N}_{\text{C-D}}$  between Glu and Phe ( $\text{TDF}_{\text{Glu-Phe}}$ , Eq. 3), that represents the lynchpin for most CSIA-AA applications in trophic ecology. As such, the central questions for ongoing CSIA-AA applications in trophic ecology have now become: What is the true variability in  $\text{TDF}_{\text{Glu-Phe}}$  and what are the underlying mechanisms controlling this variability?

In our comprehensive meta-analysis of controlled feeding experiments, we found an overall mean  $\text{TDF}_{\text{Glu-Phe}}$  value of  $6.2\% \pm 2.5\%$  across a wide range of taxa, diet types, and modes of nitrogen excretion (see Supplement 2 in Data S1 for meta-analysis table of TDF values for all trophic AA-Phe combinations). Many of the reported  $\text{TDF}_{\text{Glu-Phe}}$  values were within a fairly small range (6–8‰) that overlapped with the original  $\text{TDF}_{\text{Glu-Phe}}$  of 7.6‰ from Chikaraishi et al. (2007). Moreover, the overall mean  $\text{TDF}_{\text{Glu-Phe}}$  value is consistent with a recent meta-analysis of field-collected data for wild-caught marine consumers ( $6.6\% \pm 1.7\%$ ; Nielsen et al. 2015). Any single mean  $\text{TDF}_{\text{Glu-Phe}}$  parameter, however, inherently obscures the large variation underlying that mean (Fig. 4).

We found significant variability in  $\text{TDF}_{\text{Glu-Phe}}$  values across 70 species (317 individuals) and 88 distinct consumer–diet combinations, with a maximum of 10.4‰ for herbivorous, ammonia-producing teleost fish and a minimum of  $-0.6\%$  for herbivorous, ammonia-producing protists. Importantly, our meta-analysis strongly suggests that this variability is not simply noise, but rather is predictably linked to underlying biochemical and physiological processes. Our data indicate that phylogeny itself is not the main predictor of this variability. We found that in many cases, closely related species, or even the same species fed different diets, exhibited significantly larger ranges in  $\text{TDF}_{\text{Glu-Phe}}$  values than very distantly related phyla (Fig. 4). Below, we address in detail

two of the dominant mechanisms hypothesized to control most of the  $\text{TDF}_{\text{Glu-Phe}}$  variation in our meta-analysis: diet quality and mode of nitrogen excretion.

### *Diet quality: the master variable?*

There is a clear trend between TP and  $\text{TDF}_{\text{Glu-Phe}}$  in both our meta-analysis of  $\text{TDF}_{\text{Glu-Phe}}$  values from controlled feeding experiments, as well as two recent studies that back-calculated  $\text{TDF}_{\text{Glu-Phe}}$  values from wild marine consumers (Bradley et al. 2015, Nielsen et al. 2015). In all three studies, most primary consumers had  $\text{TDF}_{\text{Glu-Phe}}$  values between 6‰ and 8‰, often not significantly different from the original Chikaraishi et al. (2007) value of 7.6‰. In contrast, most 3<sup>+</sup> trophic-level marine consumers had significantly lower  $\text{TDF}_{\text{Glu-Phe}}$  values. One hypothesis for the underlying mechanism driving this pattern of decreasing  $\text{TDF}_{\text{Glu-Phe}}$  value with increasing TP is the influence of diet quality on consumer AA stable isotope values and thus  $\text{TDF}_{\text{Glu-Phe}}$  values. For this review, we define diet quality as the relative AA composition between diet and consumer, such that the more similar the AA composition is between diet and consumer, the higher the quality of the diet (Robbins et al. 2005, 2010). However, diet quality may also reflect the absolute protein content of the diet (Roth and Hobson 2000). Low-trophic-level consumers often feed on diets that are more compositionally different relative to their own tissues (e.g., zooplankton feeding on phytoplankton) than higher trophic-level consumers (e.g., fish feeding on other fish). Clearly, this generalization is not universal, but below we discuss how changes in diet quality across different trophic levels provide the most parsimonious explanation for the general correlation between TP and  $\text{TDF}_{\text{Glu-Phe}}$ .

It has long been understood that diet composition can influence consumer bulk stable isotope values (Hobson and Clark 1992; Robbins et al. 2005, 2010, Mill et al. 2007, Florin et al. 2011). The “diet quality” hypothesis suggests that nitrogen isotope discrimination will decrease as dietary protein quality (degree of AA similarity between diet and consumer) increases (Roth and Hobson 2000). As such, it is logical to predict that diet quality also influences individual AA nitrogen isotope fractionation patterns. McMahon et al. (2015a) was the first controlled study to

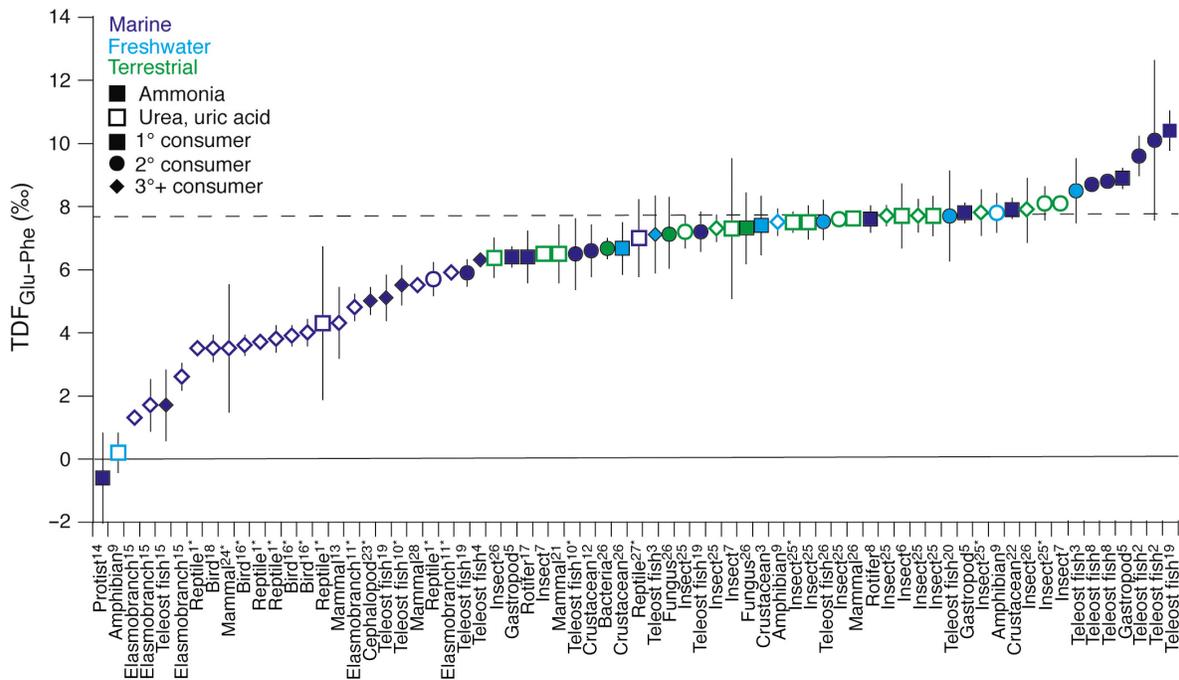


Fig. 4. Mean ( $\pm$ SD) trophic discrimination factors ( $TDF_{Glu-Phe}$ ) for a wide range of consumer–diet pairings from published controlled feeding studies and well-constrained field collections (indicated by \*).  $TDF_{Glu-Phe}$  values were calculated as the difference in trophic fractionation factors ( $\Delta^{15}N = \delta^{15}N_{consumer} - \delta^{15}N_{diet}$ ) of consumer glutamic acid and phenylalanine values. Dark blue, cyan, and green symbols represent marine, freshwater, and terrestrial consumers, respectively; filled and open symbols represent ammonia- and urea/uric acid-producing consumers, respectively; and square, circle, and diamond symbols represent primary, secondary, and tertiary (and higher) consumers, respectively. Horizontal dashed line indicates a  $TDF_{Glu-Phe}$  of 7.6‰. Superscripts indicate references: <sup>1</sup>K. Arthur, unpublished data; <sup>2</sup>Bloomfield et al. (2011); <sup>3</sup>Bowes and Thorp (2015); <sup>4</sup>Bradley et al. (2014); <sup>5</sup>Chikaraishi et al. (2007); <sup>6</sup>Chikaraishi et al. (2010a); <sup>7</sup>Chikaraishi et al. (2011); <sup>8</sup>Chikaraishi et al. (2009); <sup>9</sup>Chikaraishi et al. (2015); <sup>10</sup>Choy et al. (2012); <sup>11</sup>Dale et al. (2011); <sup>12</sup>Downs et al. (2014); <sup>13</sup>Germain et al. (2013); <sup>14</sup> Gutierrez-Rodriguez et al. (2014); <sup>15</sup>Hoen et al. (2014); <sup>16</sup>Lorrain et al. (2009); <sup>17</sup>McClelland and Montoya (2002); <sup>18</sup>McMahon et al. (2015b); <sup>19</sup>McMahon et al. (2015a); <sup>20</sup>Miller et al. (2013); <sup>21</sup>Nakashita et al. (2011); <sup>22</sup>Nakatomi et al. (2014); <sup>23</sup>Ruiz-Cooley et al. (2013); <sup>24</sup>Ruiz-Cooley et al. (2014); <sup>25</sup>Steffan et al. (2013); <sup>26</sup>Steffan et al. (2015); <sup>27</sup>Vander Zanden et al. (2013); <sup>28</sup>Zhao (2002).

conclusively show that diet quality does have a very large and systematic effect on isotopic fractionation of individual AAs in an estuarine fish (*Fundulus heteroclitus*) fed compositionally distinct diets. The study found that Phe showed minimal trophic fractionation, irrespective of AA imbalance. Conversely, there was a very strong negative relationship between the  $\Delta^{15}N_{C-D}$  of nearly all the trophic AAs (except Pro) and AA imbalance, resulting in a strong negative relationship between  $TDF_{Glu-Phe}$  and diet quality (Fig. 5). This negative relationship has subsequently been confirmed in a study that sequentially fed

commercial fish pellets to water fleas (*Daphnia magna*) to cherry shrimp (*Neocaridina heteropoda*) and guppies (*Poecilia* sp.) (Nielsen 2016). In addition, Chikaraishi et al. (2015) recently showed that by extreme manipulations of dietary composition (e.g., frogs fed carbohydrate only diets), it is also possible to obtain vastly different  $TDF_{Glu-Phe}$  values in a single consumer, further reinforcing the basic observation that diet composition strongly influences individual AA fractionation.

To understand why  $Glu \Delta^{15}N_{C-D}$ , and thus  $TDF_{Glu-Phe}$ , varies so strongly with AA composition, we again must think of the underlying

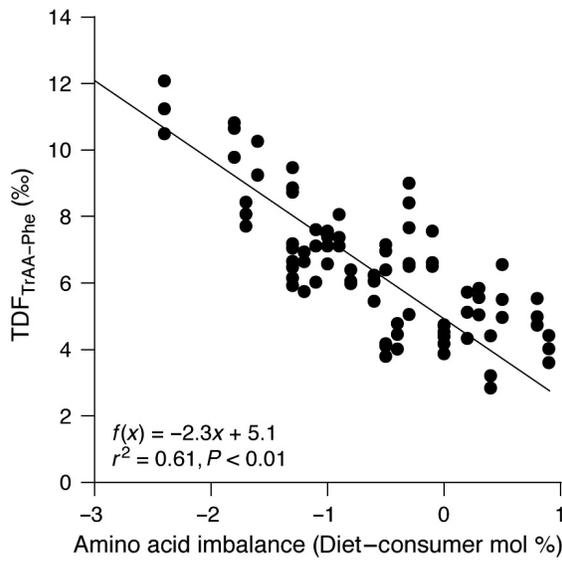


Fig. 5. The linear relationship between amino acid imbalance (individual amino acid molar percent abundance in diet minus consumer) and trophic discrimination factors (mean  $TDF_{TrAA-Phe} \pm SD$ ) of the common mummichog fish (*Fundulus heteroclitus*) fed four compositionally distinct diets (commercial pellet Veggie-Pro, commercial pellet Bio-Vita, clams, and squid; McMahon et al. 2015a). Trophic amino acids include glutamic acid, aspartic acid, alanine, isoleucine, leucine, proline, and valine all normalized to phenylalanine. Negative values for amino acid imbalance signify a lower molar percent abundance in the diet relative to the fish muscle. The  $r^2$  and  $P$  values are from reduced major axis (model II) linear regressions.

biochemical and physiological mechanisms of AA fractionation. The biochemical and AA composition of primary producers is markedly different from that of animal tissue (Roth and Hobson 2000, Clements et al. 2009), and thus, primary consumers typically need to synthesize much of their required AA pool by transamination of keto acids (Krueger and Sullivan 1984). As a result, when feeding on low-quality diets with high AA imbalance between diet and consumer requirements, a greater proportion of nitrogenous compounds available for protein synthesis are derived by sources of nitrogen that have already been enriched in  $^{15}N$  relative to the dietary AAs. Conversely, carnivores feeding on high-quality diets with AA compositions that largely match

their own tissue composition can satisfy more of their AA requirements via “direct isotopic routing” of dietary AAs (Schwarcz 1991, Ambrose and Norr 1993). Direct isotopic routing of AAs for protein biosynthesis is defined as the direct incorporation of an AA from diet in a given tissue, with no synthesis or transamination within the consumer. This is an irreversible process with no rate-limiting step and no isotopic fractionation (Braun et al. 2014). As a result, feeding on higher quality diets should result in the reduction in average  $^{15}N$  enrichment of heavily transaminating AAs (e.g., Glu) relative to consumers feeding on low-quality diets.

An additional factor associated with diet quality that may impact trophic AA  $\Delta^{15}N_{C-D}$ , and thus  $TDF_{Glu-Phe}$  value, is the balance of overall nitrogen uptake vs. excretion. Consumption and excretion rates are typically significantly lower for carnivorous fishes feeding on high-quality diets compared with herbivorous fishes (Clements et al. 2009), because the absorption efficiency of nitrogen is often higher in carnivorous species relative to herbivores (Polunin et al. 1995). As discussed in *Mode of nitrogen excretion*, the deamination of AAs during the synthesis of  $^{15}N$ -depleted ammonia and urea is a major source of trophic enrichment in the residual AA pool. Therefore, herbivores with higher excretion rates should exhibit higher fractionation in trophic AAs and thus higher  $TDF_{Glu-Phe}$  values. The net result is that lower trophic-level herbivorous or planktivorous consumers feeding on diets with larger differences in AA composition between diet and consumer tend to have higher  $TDF_{Glu-Phe}$  values than upper trophic-level carnivores.

**Mode of nitrogen excretion**

A second major observation that emerged from our meta-analysis is a clear pattern of lower  $TDF_{Glu-Phe}$  values for urea/uric acid-producing organisms relative to ammonia-producing organisms, largely driven by differences in Glu  $\Delta^{15}N_{C-D}$ , but not Phe  $\Delta^{15}N_{C-D}$  (but see terrestrial insects, *Unique systems...: Insect  $TDF_{Glu-Phe}$  values* below). Germain et al. (2013) were the first to pose the hypothesis that  $TDF_{Glu-Phe}$  value might be directly linked to mode of nitrogen excretion, after finding very low  $TDF_{Glu-Phe}$  values ( $\sim 4.3\%$ ) in harbor seals fed fish. Nielsen et al. (2015) subsequently showed a similar trend of lower

TDF<sub>Glu-Phe</sub> values for urea/uric acid-producing consumers in their large-scale (359 marine species) meta-analysis of wild marine consumers.

The explanation for why urea/uric acid producers typically have low TDF<sub>Glu-Phe</sub> values may lie in the nitrogen storage and cycling capabilities of these animals. Excess AAs in consumers cannot be stored like excess carbohydrates (as glycogen) and lipids (as triglycerides) and are therefore degraded (Campbell 1991). In this process, most excess AAs are converted to Glu via a transaminase-catalyzed reaction, which is subsequently deaminated via glutamate dehydrogenase to produce ammonia that is released into the general circulation (Fig. 2B). Ammonia is highly toxic and must be rapidly removed, either by direct excretion or by conversion to less toxic end products, such as urea or uric acid (Randall and Tsui 2002). Direct ammonia excretion is the most efficient mode of excess nitrogen removal and is characteristic of most aquatic consumers because it requires significant amounts of water to dissolve and transport ammonia (Wilkie 2002).

Key nitrogen-transferring enzymes preferentially remove <sup>14</sup>N amines during metabolism, resulting in the subsequent <sup>15</sup>N enrichment of residual animal tissue and the excretion of <sup>15</sup>N-depleted nitrogenous waste (DeNiro and Epstein 1981). Urea/uric acid biosynthesis includes all of the enzymatic steps as ammonia biosynthesis with several additional nitrogen-transferring reactions (Fig. 2D, E), providing the potential for even greater nitrogen isotope fractionation (Medina et al. 1982, Ambrose 1991). However, it is well known that the final isotope value of a biochemical reaction is dependent not only on the number of steps and associated  $\epsilon$  values (i.e., the maximal potential isotopic fractionation) but also on the relative nitrogen fluxes through branch points in the reaction chain (e.g., reviewed by Hayes 2001, Koch 2007, McCarthy et al. 2013). Germain et al. (2013) invoked this concept of variable nitrogen flux through additional branch points in the ornithine to urea pathway as likely underlying the offset in TDF<sub>Glu-Phe</sub> values for urea vs. ammonia-excreting organisms.

Urea recycling is another possible explanation for low TDF<sub>Glu-Phe</sub> values in urea/uric acid-producing consumers. Under normal growth conditions, 20–30% of biosynthesized urea is hydrolyzed by the gut microbe community to

produce <sup>15</sup>N-depleted nitrogen that can be used for de novo biosynthesis of microbial proteins or reabsorbed for the synthesis of nonessential AAs by the consumer itself (Fouillet et al. 2008). Davidson et al. (2003) hypothesized that metabolic recycling of nitrogenous materials by endosymbionts was the reason for low trophic enrichment in fluid-feeding ants. The rapidly growing recognition of the importance of the gut microbiome to both animal nutrition and molecular isotopic values suggest this as key area for future research. Importantly, the effects of nitrogen flux balance and urea recycling on AA fractionation and thus TDF<sub>Glu-Phe</sub> value are not mutually exclusive.

#### *Diet quality vs. nitrogen excretion: relative impacts on TDF values*

Our meta-analysis clearly shows that both diet quality and mode of nitrogen excretion significantly affect TDF<sub>Glu-Phe</sub> values. These processes are not mutually exclusive, and both impact the  $\Delta^{15}\text{N}_{\text{C-D}}$  of Glu by influencing the flux of nitrogen through transamination and deamination isotopic branch points. However, it is intrinsically challenging to separate the relative influences of diet quality and nitrogen excretion, simply because in most studies, low-trophic-level consumers were ammonia producers and high-trophic-level consumers were urea/uric acid producers. While sample sizes are still relatively small, our meta-analysis does suggest that the influence of diet quality may be larger than that of nitrogen excretion. In general, we found ~2‰ offset in Glu  $\Delta^{15}\text{N}_{\text{C-D}}$  between primary consumers and higher trophic-level (3<sup>+</sup>) consumers when controlled for mode of nitrogen excretion but only ~1‰ difference in Glu  $\Delta^{15}\text{N}_{\text{C-D}}$  between ammonia and urea/uric acid producers when controlled for trophic position (Fig. 6). Furthermore, Phe  $\Delta^{15}\text{N}_{\text{C-D}}$  was nearly 1‰ higher for upper trophic-level consumers compared with low-trophic-level consumers, yet there was no difference in Phe  $\Delta^{15}\text{N}_{\text{C-D}}$  between ammonia and urea/uric acid producers (Fig. 6). Given the importance of diet quality and mode of nitrogen excretion to TDF<sub>Glu-Phe</sub> variability, we argue that more targeted, mechanistic studies are needed to both quantify the fractionation of these processes and their relative importance to consumer TDF<sub>Glu-Phe</sub> value.

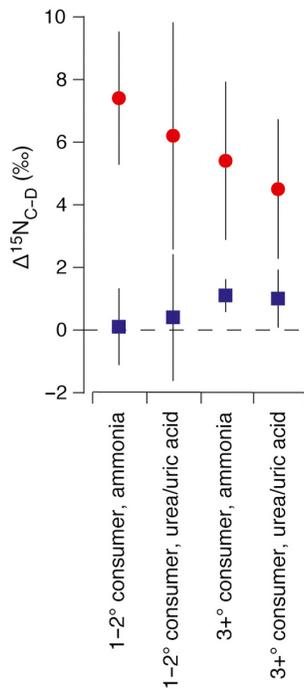


Fig. 6. Mean individual amino acid (‰ ± SD) trophic fractionation factors ( $\Delta^{15}N_{C-D} = \delta^{15}N_{consumer} - \delta^{15}N_{diet}$ ) for the trophic amino acid glutamic acid (red circles) and the source amino acid phenylalanine (blue squares) for all noninsect consumers in controlled feeding studies and well-constrained field collections, sorted by trophic level and mode of nitrogen excretion.

### UNIQUE SYSTEMS: ARE THEY EXCEPTIONS TO THE NORM?

Despite the clear patterns in individual AA  $\Delta^{15}N_{C-D}$  and  $TDF_{Glu-Phe}$  variability described above, there are groups of organisms where the common fractionation patterns for Glu and Phe do not appear to apply. The first is terrestrial insects, where  $TDF_{Glu-Phe}$  values are remarkably consistent despite highly variable  $\Delta^{15}N_{C-D}$  values of Glu and Phe, and the second is microbes, where isotopic evidence of trophic transfer can sometimes appear essentially invisible and other times appear to mimic metazoan patterns. Below, we explore the questions: Do these systems represent exceptions to the norm and furthermore do they point to critical gaps in our understanding of CSIA-AA systematics in trophic ecology?

### Insect $TDF_{Glu-Phe}$ values

Terrestrial insect  $TDF_{Glu-Phe}$  values (mean  $7.1‰ \pm 1.8‰$ ) appear to be amazingly consistent across a wide range of TPs from herbivorous aphids (TP 2) to hyperparasitoid wasps (TP 5; Fig. 4). This is in stark contrast to most marine examples where there is often a strong correlation between TP and  $TDF_{Glu-Phe}$  value, related to apparent shifts in diet quality between lower and upper trophic-level consumers. One explanation for this discrepancy is that in most insect food webs, diet quality and mode of nitrogen excretion may remain relatively constant across multiple trophic steps. For example, in an insect food web described by Steffan et al. (2013) where wasps fed on hoverflies that fed on aphids that fed on apples, beyond the primary consumer all of the trophic transfers represent one insect feeding on another. We hypothesize that perhaps insect food webs are more akin to a multitrophic position food web of zooplankton where there are no large systematic shifts in diet quality or mode of nitrogen excretion and thus no large changes in  $TDF_{Glu-Phe}$  (Fig. 7A). If correct, insect food webs would remain consistent with the underlying mechanisms proposed in *Variability in Trophic Discrimination Factors*.

However, in several respects,  $TDF_{Glu-Phe}$  patterns reported in insects depart substantially from the framework of fractionation described in *Nitrogen Isotope Fractionation in Amino Acids*. First, the observed linkage to mode of nitrogen excretion observed across the meta-analysis does not seem to apply to insects. Insects produce uric acid, yet their  $TDF_{Glu-Phe}$  values ( $7.1‰ \pm 1.8‰$ ) were significantly higher than we observed for all other urea/uric acid-producing consumers in our meta-analysis (mean  $4.4‰ \pm 1.9‰$ ; Fig. 4). It remains to be explained why insect Glu  $\Delta^{15}N_{C-D}$  patterns for uric acid-producing insects deviate from the patterns observed for most other urea/uric acid-producing consumers.

An even more fundamental departure for insects lies in the trophic fractionation patterns of the canonical source AA Phe. Large negative Phe  $\Delta^{15}N$  values have been observed in beetles, aphids, and lacewings ( $-1.6‰ \pm 2.4‰$ ; Table 1), suggesting that Phe was in fact not behaving as a source AA for these insects and thus not serving as a proxy for  $\delta^{15}N_{baseline}$ . Furthermore, in essentially all of these cases where Phe

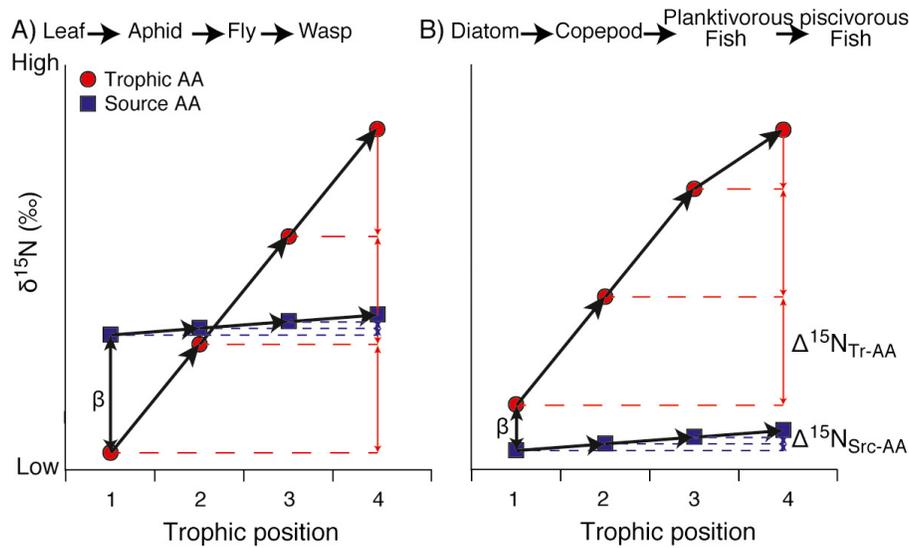


Fig. 7. Schematic representations of the relationships between the  $\delta^{15}\text{N}$  value of trophic amino acids (Tr-AA; red circles), source amino acids (Src-AA; blue squares), and relative trophic position for a simple food chain with a single isotope baseline.  $\beta$  represents the difference in  $\delta^{15}\text{N}$  between primary producer Tr-AA and Src-AA value, and  $\Delta^{15}\text{N}_{\text{Tr-AA}}$  and  $\Delta^{15}\text{N}_{\text{Src-AA}}$  represent the trophic fractionation during a single trophic transfer for the trophic and source amino acids, respectively. Panel A reflects a typical terrestrial insect food chain from plant leaves to wasps with a negative  $\beta$  value for primary producers and relatively constant  $\Delta^{15}\text{N}_{\text{Tr-AA}}$  values across all trophic transfers. Panel B reflects a typical aquatic food chain from diatoms to carnivorous fishes showing a characteristic decrease in  $\Delta^{15}\text{N}_{\text{Tr-AA}}$  between planktivorous and piscivorous fishes associated with higher diet quality. Figure modified from the original conception in Chikaraishi et al. (2009).

$\Delta^{15}\text{N}_{\text{C-D}}$  is substantially negative, Glu  $\Delta^{15}\text{N}_{\text{C-D}}$  is correspondingly positive, such that together  $\text{TDF}_{\text{Glu-Phe}}$  is approximately 7.6‰ for all species (Table 1). This implies a direct linkage between Glu and Phe  $\Delta^{15}\text{N}$ , which again would fundamentally depart from our understanding of trophic and source AA fractionation.

One hypothesis for the enhanced fractionation in Phe for these insects is related to the relative flux of Phe through transamination (fractionating) and hydroxylation (nonfractionating) pathways. The diphenols produced during the metabolism of the aromatic AAs Phe and Tyr have important functions as cross-linking structures for the sclerotization of insect cuticulae (Andersen et al. 1996), such that increased Phe transamination might accompany increased demand in aromatic AA breakdown for molting insects. However, this mechanism should lead to a positive fractionation in Phe; there are currently no mechanistic explanations to link possible increased breakdown in Phe to depletion of  $^{15}\text{N}$  of the remaining Phe pool. Another explanation for negative Phe  $\Delta^{15}\text{N}_{\text{C-D}}$

values is direct routing of alternate Phe sources in selected insects/environments, for example, from soil or gut microbes (Engel and Moran 2013). This explanation could also explain the simultaneous, coupled changes in Glu  $\Delta^{15}\text{N}_{\text{C-D}}$  values, assuming microbes were synthesizing Phe with nitrogen from the central nitrogen pool linked to Glu. However, this hypothesis still does not provide an explanation for the negative  $\Delta^{15}\text{N}_{\text{C-D}}$  values of Phe. A third explanation could simply be that the average Phe  $\delta^{15}\text{N}$  values for diets used to calculate Phe  $\Delta^{15}\text{N}_{\text{C-D}}$  may not reflect the dietary source of Phe in the heterogeneous diets fed to these insects. Clearly, this is an area that requires further mechanistic research, for both the importance of insects in terrestrial food webs and our general knowledge about how AA metabolism impacts AA  $\Delta^{15}\text{N}_{\text{C-D}}$ .

**Microbial food webs: isotopically visible or invisible?**

In contrast to most metazoans, AA fractionation patterns in microbial-dominated food webs present clear exceptions to the typical trophic

Table 1. Trophic fractionation factors (mean  $\Delta^{15}\text{N}_{\text{C-D}} \pm \text{SD}$  in ‰) for glutamic acid (Glu) and phenylalanine (Phe) as well as resulting  $\text{TDF}_{\text{Glu-Phe}}$  values (mean  $\pm \text{SD}$  in ‰) for terrestrial insects reared in controlled feeding experiments.

Consumer	Glu $\Delta^{15}\text{N}_{\text{C-D}}$	Phe $\Delta^{15}\text{N}_{\text{C-D}}$	$\text{TDF}_{\text{Glu-Phe}}$
<b>Butterflies/Moths</b>			
<i>Pieris rapae</i> (n = 4) <sup>6,7</sup>	7.7 ± 0.9	0.2 ± 1.0	7.5 ± 1.4
<i>Spodoptera frugiperda</i> (n = 4) <sup>25</sup>	7.8 ± 0.4	0.3 ± 0.2	7.5 ± 0.5
<i>Plodia interpunctella</i> (n = 4) <sup>26</sup>	9.8 ± 1.8	1.7 ± 1.3	8.1 ± 0.6
<b>Wasps</b>			
<i>Bothriothorax</i> sp. (n = 4) <sup>25</sup>	7.0 ± 1.8	-0.9 ± 1.4	7.8 ± 0.7
<i>Pachyneuron albutius</i> (n = 4) <sup>25</sup>	7.7 ± 1.5	0.4 ± 1.5	7.3 ± 0.4
<b>Flies</b>			
<i>Eupeodes</i> sp. (n = 4) <sup>25</sup>	7.9 ± 1.9	-0.1 ± 1.9	8.1 ± 0.5
<b>Aphids</b>			
<i>Acyrtosiphon pisum</i> (n = 3) <sup>25</sup>	4.6 ± 0.8	-3.1 ± 0.8	7.7 ± 0.6
<i>Aphidoidea</i> sp. (n = 1) <sup>7</sup>	5.9	-0.5	6.5
<i>Aphis pomi</i> (n = 4) <sup>25</sup>	6.8 ± 1.8	-0.7 ± 1.9	7.5 ± 0.3
<b>Lacewings</b>			
<i>Chrysopa nigricornis</i> (n = 14) <sup>25</sup>	5.7 ± 3.4	-1.8 ± 3.3	7.6 ± 0.4
<b>Beetles</b>			
<i>Harmonia axyridis</i> (n = 1) <sup>7</sup>	6.1	-2.0	8.1
<i>Dermestes</i> sp. (n = 3) <sup>26</sup>	5.3 ± 0.2	-2.4 ± 1.6	7.7 ± 1.4
<i>Tribolium castaneum</i> (n = 3) <sup>26</sup>	7.6 ± 1.7	1.3 ± 1.2	6.3 ± 0.6

Notes: TDF, trophic discrimination factor. Superscripts refer to references according to Fig. 4 legend.

and source AA fractionation patterns described in *Nitrogen Isotope Fractionation in Amino Acids*. Three general patterns of AA fractionation in microbes have been identified. Hannides et al. (2009) demonstrated that AA  $\delta^{15}\text{N}$  values change during degradation of ocean suspended particles consistent with an external hydrolysis (Raleigh fractionation) model, such that the  $\delta^{15}\text{N}$  values of both Glu and Phe (as well as all other AAs) increase evenly with microbial degradation, resulting in  $\text{TP}_{\text{CSIA}}$  values that were not significantly different from those expected for pure herbivores ( $\text{TP} = 2$ ). A second pattern of microbial AA fractionation also does not follow the typical metazoan trophic and source AA distinctions. Gutierrez-Rodriguez et al. (2014) showed that protist consumers reared on microalgae in a controlled chemostat experiment exhibited no significant enrichment in all AAs but Ala and Gly, resulting in the lowest  $\text{TDF}_{\text{Glu-Phe}}$  values ( $-0.6\text{‰} \pm 1.4\text{‰}$ ) of any consumer–resource relationship in our meta-analysis. This pattern of only selected AA change with microbial heterotrophy has also been observed in multiple other studies (e.g., Fogel and Tuross 1999, McCarthy et al. 2007, Calleja et al. 2013), suggesting that microbes may often incorporate most AAs via

direct isotope routing with minimal trophic fractionation. Finally, a recent study by Steffan et al. (2015) demonstrates a third pattern, in which bacteria can mimic classic metazoan trophic and source patterns. In this study, bacteria fed on high protein yeast extract exhibited  $\text{TDF}_{\text{Glu-Phe}}$  values of  $6.6\text{‰} \pm 0.3\text{‰}$ , similar to metazoan consumers. Steffan et al. (2015) suggested that when bacteria are fed the same diets as animals, they are trophic analogs to animals. Recent work further supports these findings, indicating that bacteria grown on pure (free) AAs show isotopic fractionation patterns consistent with typical trophic and source AAs (Yamaguchi 2013).

We hypothesize that the central issue underlying these divergent observations in microbial AA fractionation is that unlike metazoan consumers, bacteria and protists are able to use a wide variety of both inorganic and organic nitrogen sources. As a result, microbes can derive AAs via three distinct mechanisms: (1) de novo synthesis of all AAs from inorganic nitrogen (e.g., Macko et al. 1987, Maki et al. 2014), (2) direct or “salvage” incorporation of unaltered dietary AAs (e.g., Fogel and Tuross 1999, McCarthy et al. 2007, Calleja et al. 2013), and (3) trophic resynthesis and transamination of trophic but not source

AAs (Steffan et al. 2015). Hoch et al. (1996) provided direct experimental evidence for this idea, showing that bulk  $\Delta^{15}\text{N}_{\text{C-D}}$  values in protists consuming bacteria could vary widely, from high values typical of metazoan consumers to nearly “invisible” values, depending on the sources and extent of nitrogen recycling. The resulting potential diversity and complexity of  $\text{TDF}_{\text{Glu-Phe}}$  values in microbial heterotrophy forms the basis for the  $\Sigma V$  parameter now used to assess microbial AA resynthesis in detrital materials (McCarthy et al. 2007). Overall, while the diverse AA fractionation potential of microbes seems clear, a predictive framework that can be used across diverse environments remains lacking. Given the critical roles microbes play in biogeochemical cycling, food web structure, and energy transfer, we suggest that research aimed at a predictive understanding of AA  $\delta^{15}\text{N}$  fractionation during microbial heterotrophy is a key area of future research.

#### INCORPORATING TDF VARIABILITY INTO TROPHIC ECOLOGY

Among the conclusions from our comprehensive meta-analysis of controlled feeding studies, two observations about  $\text{TDF}_{\text{Glu-Phe}}$  values stand out as having broad implications for CSIA-AA in trophic ecology: (1) There is very significant variability in  $\text{TDF}_{\text{Glu-Phe}}$  values about the mean, and (2) this variability appears to be systematic, reflecting predictable patterns of trophic AA fractionation associated with diet quality (AA imbalance) and mode of nitrogen excretion. To date, very few CSIA-AA studies have attempted to explicitly account for potential  $\text{TDF}_{\text{Glu-Phe}}$  variability in estimates of  $\text{TP}_{\text{CSIA}}$ , in part because we are only now beginning to realize that the potential range in  $\text{TDF}_{\text{Glu-Phe}}$  values (Fig. 4) is also systematic. Of the 60 environmental application studies that calculated  $\text{TP}_{\text{CSIA}}$  (Fig. 1 inset), almost all (92%) used a fixed  $\text{TDF}_{\text{Glu-Phe}}$  value of either 7‰ or 7.6‰ in Eq. 2 (i.e., the most common values based on McClelland and Montoya 2002 or Chikaraishi et al. 2009). This approach is likely accurate for studies dealing with food webs in which all consumers have the same mode of nitrogen excretion and relative diet quality (e.g., Steffan et al. 2013, Chikaraishi et al. 2014). However, given the clear impacts of diet

quality and mode of nitrogen excretion on AA fractionation, we suggest that the accuracy of  $\text{TP}_{\text{CSIA}}$  estimates can be substantially improved by moving to new approaches that directly incorporate variability in  $\text{TDF}_{\text{Glu-Phe}}$  values into  $\text{TP}_{\text{CSIA}}$  estimates (Fig. 7B), particularly in systems where significant changes in diet quality and/or mode of nitrogen excretion take place within a food web (e.g., Lorrain et al. 2009, Dale et al. 2011, Choy et al. 2012, Germain et al. 2013, Ruiz-Cooley et al. 2013, 2014, Matthews and Ferguson 2014, McMahon et al. 2015b).

Germain et al. (2013) first proposed a multi-TDF approach that explicitly incorporated separate  $\text{TDF}_{\text{Glu-Phe}}$  values for key transitions in mode of nitrogen excretion across a food web. Our meta-analysis indicates that this multi-TDF approach should also be extended to key transitions in diet quality as well as mode of nitrogen excretion, resulting in a more general multi-TDF equation:

$$\text{TP}_{\text{CSIA-multi-TDF}} = (x + 1) + \left[ \frac{\delta^{15}\text{N}_{(\text{Glu})} - \delta^{15}\text{N}_{(\text{Phe})} - x \times \text{TDF}_1 - \beta}{\text{TDF}_2} \right] \quad (4)$$

where  $\text{TDF}_1$  represents the  $\text{TDF}_{\text{Glu-Phe}}$  value typical of lower trophic-level organisms (e.g., Chikaraishi et al. 2007),  $x$  is the number of trophic levels influenced by  $\text{TDF}_1$ ,  $\beta$  is the same as Eq. 2, and  $\text{TDF}_2$  reflects a key shift in mode of nitrogen excretion and/or diet quality.

A couple of recent papers illustrated how applying this multi-TDF approach can significantly improve  $\text{TP}_{\text{CSIA}}$  estimates in top predators and urea/uric acid producers. For example, Choy et al. (2012) found that  $\text{TP}_{\text{CSIA}}$  estimates of zooplanktivorous lanternfishes (family Myctophidae) calculated from a single- $\text{TDF}_{\text{Glu-Phe}}$  value of 7.6‰ aligned well with expected TP values from 361 published stomach content records. However, the similarly calculated  $\text{TP}_{\text{CSIA}}$  values of piscivorous dragonfishes (family Stomiidae) were a full trophic level lower than expected from 73 published stomach content records. McMahon et al. (2015a) found that recalculating dragonfish  $\text{TP}_{\text{CSIA}}$  using a multi- $\text{TDF}_{\text{Glu-Phe}}$  equation that accounted for the expected reduction in  $\text{TDF}_{\text{Glu-Phe}}$  associated with the high diet quality trophic transfer between lanternfishes and dragonfishes significantly

improved the accuracy of the  $TP_{CSIA}$  calculation (Fig. 8). Similarly, McMahon et al. (2015b) showed that utilizing a multi-TDF approach that accounted for diet quality and uric acid production significantly improved  $TP_{CSIA}$  estimates of wild penguins (Fig. 8). However, even the multi-TDF<sub>Glu-Phe</sub> approach still appeared to underestimate wild penguin  $TP_{CSIA}$  values. Several recent studies on marine mammals similarly found that while a multi-TDF equation improved estimates of  $TP_{CSIA}$ , the calculated  $TP_{CSIA}$  values were still ecologically unrealistic (Matthews and Ferguson 2014, Ruiz-Cooley et al. 2014). This could reflect issues with the specific TDF<sub>Glu-Phe</sub> values used or biases in TP estimates from conventional TP metrics (e.g., stomach content analysis and feeding observations). Nonetheless, these examples illustrate the potential advantages, as well as challenges, of taking diet composition and mode of nitrogen excretion into account when calculating the  $TP_{CSIA}$  of consumers.

A complementary approach to improving the accuracy and precision of  $TP_{CSIA}$  estimates is to use averages of multiple trophic and source AAs when calculating TDF values (e.g., McCarthy et al. 2007, Bradley et al. 2015, Nielsen et al. 2015). McCarthy et al. (2007) first proposed a  $TP_{CSIA}$  equation based on averages of multiple AAs for use in detrital materials, in which either complex analytical matrixes or uncertainties related to degradation might potentially affect individual AA  $\delta^{15}N$  values. Nielsen et al. (2015) found that modeled uncertainties in  $TP_{CSIA}$  estimates significantly decreased when increasing the number of trophic and source AAs in the calculation. However, it is important to note that care must be taken when choosing appropriate AAs. For instance, Gly and Ser have been shown to exhibit highly variable trophic fractionation across taxa and diet types, particularly for upper trophic-level metazoans (Chikaraishi et al. 2009, 2015, McMahon et al. 2015b, Nielsen et al. 2015, Steffan et al. 2015). Furthermore, turnover rates can vary substantially among individual AAs (Bradley et al. 2014, Downs et al. 2014). While these differences may prove useful in determining timing of diet switches or movement ecology in consumers, they will certainly pose challenges for interpreting resource utilization using AAs at varying stages of isotopic equilibrium with diet. This is an area of active research that deserves

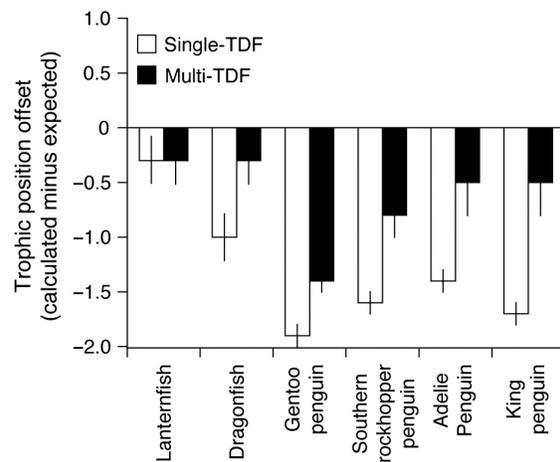


Fig. 8. Examination of trophic position offsets between literature values of expected trophic position (typically from extensive gut content analyses, bulk stable isotopes, and feeding observations) and trophic positions calculated with single- and multi-trophic discrimination factor (TDF) compound-specific equations. Compound-specific trophic positions were calculated with consumer trophic amino acid ( $\delta^{15}N_{Glu}$ ) and source amino acid ( $\delta^{15}N_{Phe}$ ) values and either a single-TDF<sub>Glu-Phe</sub> value of 7.6‰ for all trophic transfers (single-TDF; open bars), or a multi-TDF<sub>Glu-Phe</sub> equation using 7.6‰ for the first trophic transfer, and mean TDF<sub>Glu-Phe</sub> values for the predicted diet quality and mode of nitrogen excretion (multi-TDF; filled bars). Amino acid  $\delta^{15}N$  data, expected trophic positions, and single-TDF trophic position estimates for lanternfishes ( $n = 5$ ) and ( $n = 5$ ) were from Choy et al. (2012), while the multi-TDF trophic position estimates were calculated in McMahon et al. (2015a). Amino acid  $\delta^{15}N$  data, expected trophic positions, and single-TDF trophic position estimates for King ( $n = 3$ ), northern rockhopper ( $n = 2$ ), Adélie ( $n = 3$ ), and southern rockhopper penguins ( $n = 2$ ) came from Lorrain et al. (2009), while the multi-TDF trophic position estimates were calculated in McMahon et al. (2015b). Amino acid  $\delta^{15}N$  data, expected trophic positions, and both single-TDF and multi-TDF trophic position estimates for Gentoo penguins ( $n = 5$ ) came from McMahon et al. (2015b).

significant attention in the hopes of improving the accuracy and precision of  $TP_{CSIA}$  estimates.

Even among the canonical trophic and source AAs, the “best” choices will ultimately depend on the abundance of individual AAs in studied

organisms and, to some degree, the laboratory's analytical system. Different derivatization/separation schemes differ in what subset of total AAs can be reliably quantified, based on a combination of derivative chemistry and column separation (Chikaraishi et al. 2010b). Currently, two derivative systems account for most published AA  $\delta^{15}\text{N}$  data: *N*-pivaloyl isopropyl esters (Pv/iPr; e.g., Chikaraishi et al. 2007, 2009) and *N*-trifluoroacetyl isopropyl esters (TFA/iPr; e.g., McCarthy et al. 2007, Popp et al. 2007). A third derivatization method, based on chloroformate derivatives (Walsh et al. 2014), is increasingly being used for AA  $\delta^{13}\text{C}$  data but is currently not widely used for AA  $\delta^{15}\text{N}$  data due to issues with pH dependent fractionation of Glu (Y. Chikaraishi, *personal communication*). Ultimately, it is the chromatographic separation, determined by the interaction between derivative and GC column that determines the quantifiable AAs (Chikaraishi et al. 2010b). For example, while the Pv/iPr method can resolve Thr on many columns (Chikaraishi et al. 2010b), in natural samples it is rarely reported, because it is poorly resolved when using typical separations optimized for Glu and Phe. Given that any derivative/separation system represents a sample-dependent compromise in the resolution of 15 individual compounds, the "best" group of source and trophic AA will always be to some degree sample and analysis dependent.

## SUMMARY AND FUTURE DIRECTIONS

The goal of this review was to both quantify the variability in  $\text{TDF}_{\text{Glu-Phe}}$  values that characterize consumer–resource relationships and explore potential underlying biochemical drivers as a starting point for refining calculations of  $\text{TP}_{\text{CSIA}}$  using AA  $\delta^{15}\text{N}$  values. In the broadest sense, this review reaffirms the notion that classifying AAs into heavily fractionating trophic AAs (e.g., Glu, Asp, Ala, Pro, Ile, Leu, Val) and minimally fractionating source AAs (e.g., Phe, Met, Lys) is a useful framework for characterizing the inherent trophic transfer information retained in their  $\delta^{15}\text{N}$  values. Together, these AAs can provide a powerful tool to estimate  $\text{TP}_{\text{CSIA}}$  that is internally indexed to  $\delta^{15}\text{N}_{\text{baseline}}$  values. However, our review also clearly shows that the degree of fractionation among these AAs (Fig. 3)

is far from universal, resulting in a substantial range in  $\text{TDF}_{\text{Glu-Phe}}$  values among consumers (Fig. 4). Careful consideration of the biochemical and physiological mechanisms driving AA fractionation is therefore critical to developing the most accurate framework for applications of CSIA-AA to trophic ecology.

Our meta-analysis revealed two dominant variables that appear to drive much of the observed variability in  $\text{TDF}_{\text{Glu-Phe}}$  values across a diverse suite of consumer–resource relationships: diet quality and mode of nitrogen excretion. Consumers feeding on high-quality diets with small AA imbalances between diet and consumer tend to have significantly lower  $\text{TDF}_{\text{Glu-Phe}}$  values than consumers feeding on low-quality diets. Similarly, urea/uric acid-producing consumers also exhibit significantly lower  $\text{TDF}_{\text{Glu-Phe}}$  values than their ammonia-producing counterparts. These patterns are largely driven by variation in the fractionation of trophic AAs associated with the flux of nitrogen through isotopic branch points in metabolic processing of these AAs. We further suggest that a combination these two drivers reflects the most parsimonious explanation for the now widely observed correlation between  $\text{TDF}_{\text{Glu-Phe}}$  and TP in noninsect systems.

We end our review with a traditional call for future research, but in this case a very targeted one. To realize the full potential of CSIA-AA approaches in trophic ecology, we argue that the scientific community must explicitly embrace the variability in  $\text{TDF}_{\text{Glu-Phe}}$  values. The results of our meta-analysis and recent case studies make it clear that while a single- $\text{TDF}_{\text{Glu-Phe}}$  value may work well for consumers feeding within a food web of generally similar diet quality and mode of nitrogen excretion, substantial increases in the accuracy and precision of  $\text{TP}_{\text{CSIA}}$  estimates can be achieved using new approaches that use multiple TDF values (potentially averaged across multiple AAs) that take into account systematic variability in TDF values (Figs. 7, 8). To do this, we need a robust framework for incorporating TDF variation into  $\text{TP}_{\text{CSIA}}$  calculations. Clearly, developing this new framework of multi-TDF calculations of  $\text{TP}_{\text{CSIA}}$  will not be trivial. This will require more accurate accounting of important transitions in diet quality and mode of nitrogen excretion within food webs, as well as careful cost–benefit

analysis of potential improvements in the accuracy of  $TP_{CSIA}$  calculations relative to the current approach. Furthermore, this framework needs to be fully grounded by an understanding of the biochemical and physiological factors controlling individual AA nitrogen isotope fractionation. These improvements should usher in the next major advancement in studies of resource acquisition and allocation, trophic dynamic, and food web architecture using CSIA-AA.

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