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HIGHLIGHTED STUDENT RESEARCH

Meta-analysis of amino acid stable nitrogen isotope ratios for estimating trophic position in marine organisms

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Abstract Estimating trophic structures is a common approach used to retrieve information regarding energy pathways, predation, and competition in complex ecosystems. The application of amino acid (AA) compound-specific nitrogen (N) isotope analysis (CSIA) is a relatively new method used to estimate trophic position (TP) and feeding relationships in diverse organisms. Here, we conducted the first meta-analysis of δ^{15}N AA values from measurements of 359 marine species covering four trophic levels, and compared TP estimates from AA-CSIA to literature values derived from food items, gut or stomach content analysis. We tested whether the AA trophic enrichment factor (TEF), or the ^{15}N enrichment among different individual AAs is constant across trophic levels and whether inclusion of δ^{15}N values from multiple AAs improves TP estimation. For the TEF of glutamic acid relative to phenylalanine (Phe) we found an average value of 6.6 ‰ across all taxa, which is significantly lower than the commonly applied 7.6 ‰. We found that organism feeding ecology influences TEF values of several trophic AAs relative to Phe, with significantly higher TEF values for herbivores compared to omnivores and carnivores, while TEF values were also significantly lower for animals excreting urea compared to ammonium. Based on the comparison of multiple model structures using the metadata of δ^{15}N AA values we show that increasing the number of AAs in principle improves precision in TP estimation. This meta-analysis clarifies the advantages and limitations of using individual δ^{15}N AA values as tools in trophic ecology and provides a guideline for the future application of AA-CSIA to food web studies.

Keywords Compounds-specific isotope analysis · Food webs · Trophic enrichment factor · Trophic ecology

Introduction

A continuous challenge in ecology is to predict and estimate trophic structures in complex food webs. Accurate knowledge of trophic interactions is critical to the understanding of energy pathways, predation and competition within ecosystems. Predictable nitrogen-15 (^{15}N) enrichment of N isotopic composition with each trophic position (TP) has been extensively used to trace pathways of organic material and to understand food web structure in biological systems (Boecklen et al. 2011; McCutchan et al. 2003; Post 2002). However, in bulk tissue or whole animal analyses, ^{15}N enrichment of consumer tissue relative to diet is often variable within a trophic level (TL) due to confounding biotic and abiotic factors (Martínez del Rio et al. 2009; Vanderklift and Ponsard 2003). Difficulties may also arise in accurately estimating the isotopic composition of N sources at the base of the food web (isotopic baseline) (Post 2002), since ecosystem biogeochemistry is dynamic and multiple nutrient sources available to phytoplankton can have distinct isotopic values that also vary

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temporally (Grey et al. 2001; Rolff 2000) and spatially (Mackenzie et al. 2011; McMahon et al. 2013). Even when isotopic baseline sampling is possible it may not represent consumer dietary incorporation due to preferential feeding and/or assimilation. To overcome these limitations, the application of amino acid (AA) compound-specific N isotope analysis (AA-CSIA) has been recommended to estimate the TP of organisms (Chikaraishi et al. 2009; McClelland and Montoya 2002; Popp et al. 2007).

The advantage of AA-CSIA is that AA stable N isotope ratios ($\delta^{15}N$ values) in a consumer encode information about both TP and isotopic baseline. The $\delta^{15}N$ values of some AAs [i.e. source AAs sensu Popp et al. (2007)] in consumers are very similar to those in the producers that synthesized them and thus carry information about the N source of the marine environment. The $\delta^{15}N$ values of other AAs [i.e. trophic AAs sensu Popp et al. (2007)] become enriched in ^{15}N relative to source AAs with each trophic transfer (McClelland and Montoya 2002). The $\delta^{15}N$ values of source AAs in consumers thus provide a measurement of isotopic baseline of the nitrogenous nutrients assimilated into that consumer's tissue, while the difference in $\delta^{15}N$ values of trophic AAs relative to source AAs provides an estimate of the animal's TP (Chikaraishi et al. 2007; McClelland et al. 2003; Popp et al. 2007; Schmidt et al. 2004). The dual information in a consumer's tissue renders AA-CSIA applicable for assessing a variety of ecological questions and can allow for more precise TP estimation compared with bulk tissue $\delta^{15}N$ values across regions with different isotopic baselines (Choy et al. 2012; Dale et al. 2011). Understanding spatial variation in consumer source AA $\delta^{15}N$ values also provides insight into species' segregation and migration patterns (Madigan et al. 2014; Ruiz-Cooley et al. 2013; Seminoff et al. 2012).

Chikaraishi et al. (2009) proposed that TP estimation can be expressed according to

$$\mathrm{TP}_{x/y} = \frac{\delta^{15}N_x - \delta^{15}N_y - \beta_{x/y}}{\Delta_x - \Delta_y} + 1, \quad (1)$$

where $\beta_{x/y}$ is the difference between the $\delta^{15}N$ values of trophic AA(s) x and source AA(s) y in primary producers and Δ_x and Δ_y are the ^{15}N enrichment factors with each trophic transfer for AA(s) x and y, respectively. This formulation relies on the fact that the trophic AA is enriched in ^{15}N relative to the source AA for each trophic transfer, and the trophic enrichment factor (TEF; $\mathrm{TEF}_{x-y} = \Delta_x - \Delta_y$) is the ^{15}N diet to consumer enrichment in the trophic AA relative to source AA (Chikaraishi et al. 2009). Chikaraishi et al. (2009) recommended the use of the $\delta^{15}N$ values of the trophic AA glutamic acid (Glu) and source AA phenylalanine (Phe) for assessing TP due to the relatively large and constant ^{15}N enrichment in Glu relative to Phe with each trophic transfer ($\mathrm{TEF}_{\mathrm{Glu\text{-}Phe}} = 7.6$ ‰) and consistent β value ($\beta_{\mathrm{Glu/Phe}} = 3.4$ ‰) found in marine primary producers. Several studies have applied Eq. 1 for TP estimation using differences in the $\delta^{15}N$ values of Glu and Phe (Chikaraishi et al. 2009; Hannides et al. 2009; O'Malley et al. 2012); however, in principle any combination of $\delta^{15}N$ values of single or multiple trophic and source AAs should yield an estimate of TP (e.g. Popp et al. 2007; Hannides et al. 2009; Sherwood et al. 2011; Décima et al. 2013). Indeed, it has been suggested that TP models based on multiple trophic and source $\delta^{15}N$ AA values improve TP estimation (Décima et al. 2013; Hoen et al. 2014; Sherwood et al. 2011). Previous multiple AA TP estimation models typically include $\delta^{15}N_{\mathrm{Glu}}$ and $\delta^{15}N_{\mathrm{Phe}}$ values; however, a general assessment of other potential AA candidates is currently lacking.

Accurate estimation of TP using AA-CSIA remains nevertheless challenging, as it relies on accurate estimates of both TEF and β-values. Recent studies have indicated that TP of marine organisms may be under-estimated, especially at higher TL, and it has been speculated that $\mathrm{TEF}_{\mathrm{Glu\text{-}Phe}}$ is variable and lower than the proposed value of 7.6 ‰ (Dale et al. 2011; Hoen et al. 2014; McCarthy et al. 2013; Olson et al. 2010). Increasing organism TP may not be the sole reason for variation in TEF, as it may also be influenced by other factors such as food quality (Bloomfield et al. 2011; Hoen et al. 2014) and N metabolism (Dale et al. 2011; Germain et al. 2013). Increased protein reworking during intake of poor-quality food sources (i.e. low protein) to account for a dietary imbalance may explain higher ^{15}N AA enrichment in herbivore compared to carnivore consumers (Bloomfield et al. 2011; Hoen et al. 2014). Differences in the metabolic N pathway between urea (CH_4N_2O) and ammonia (NH^+_4) production may also influence AA ^{15}N enrichment, as observed for marine mammals (Germain et al. 2013), sharks and rays (Dale et al. 2011).

Here we present a meta-analysis of $\delta^{15}N$ AA values including studies across multiple TLs to evaluate the magnitude of TEF values and to test the broad-scale application of AA-CSIA as a tool to predict TP of marine animals. Specifically we ask the following questions:

1. Is ^{15}N enrichment in trophic AAs relative to source AAs constant across TP?
2. Does feeding ecology through variable dietary input and form of N excretion affect the value of TEF?
3. Which AA $\delta^{15}N$ values are the most suitable for TP estimation?
4. Are TP estimates which incorporate multiple AA $\delta^{15}N$ values more precise than a model using single trophic and source AA $\delta^{15}N$ values?

We compiled available AA $\delta^{15}N$ values from the literature and compared TP estimation based on AA $\delta^{15}N$ values with

literature estimates ($TP_{literature}$) based on diet items or from stomach or gut content analysis. TP estimation from stomach or content analysis has been used for decades (Hynes 1950; Hyslop 1980) and is considered as the best comparison to TP calculated from AA-CSIA despite known uncertainties and potential biases associated with stomach or gut content analysis (Rindorf and Lewy 2004). We constructed a modelling framework using $\delta^{15}N$ values from multiple AAs, and further validated uncertainty associated with each parameter ($\delta^{15}N$ AA values, TEF, β). Our analysis across four TLs showed the advantage of incorporating the information from $\delta^{15}N$ values of multiple AAs for elucidating trophic interactions in marine food webs. We also show that the TEF value is the most sensitive parameter to TP estimation error, and that the value of TEF may vary significantly due to differences in feeding ecology and the form of N excretion.

Materials and methods

Data collection and statistical analysis

We compiled $\delta^{15}N$ AA-CSIA data from available literature sources which included 30 studies and 359 measurements of individuals or pooled marine samples. The metadata covered measurements across four TLs from muscle tissue or whole animals, and included primary producers, invertebrates (zooplankton, corals, sea slugs, shrimps, crabs, clams, gastropods, lobsters and cephalopods), bony fishes, elasmobranchs, penguins, turtles and seals (list of studies are provided in ESM Appendix A). In the analysis of the form of N excretion we also included measurements from blood samples from the studies by Germain et al. (2013) and Lorrain et al. (2009). Measurements from fish scales were also included from a single study (Chikaraishi et al. 2014), as the authors found no differences in AA N isotope composition between scales and muscle tissue. Species were classified as herbivore, omnivore or carnivore based on their reported feeding ecology and based on the form of N excretion assigned as either CH_4N_2O or NH^+_4 (ESM Appendix A). All organisms were assigned an estimated $TP_{literature}$ value based on their primary food resource from literature estimates. Primary producers received a $TP_{literature}$ of 1, herbivore consumers a $TP_{literature}$ of 2, omnivore species an intermediate $TP_{literature}$ of 2.5. $TP_{literature}$ values of higher TL species were taken from the associated study, if reported, or other relevant literature sources, based on SCA or ecopath models (Froese and Pauly 2012). We used $TP_{literature}$ estimates only when the stomach contents of a sufficiently large number of organisms were available as determined from publications on the gut contents of each species. Quantification of the TP of a marine organism through stomach or gut content analysis is not always straightforward and several critical reviews of this subject exist (Baker et al. 2014; Cortés 1997; Hynes 1950; Hyslop 1980). Uncertainty of $TP_{literature}$ estimates vary between individual organisms and for several species included in this meta-analysis, and due to the approach applied in most ecopath models the standard error (SE) may be close to 0.5 TP (Froese and Pauly 2012). Uncertainties are likely higher for species where diet knowledge is scarce and rarely do sufficient data exist to evaluate ontogenetic changes in diet (Dale et al. 2011; Graham et al. 2007). Although imperfect, we consider that the output from these routines are the best available estimates of fractional TP from natural populations of marine organisms. We focussed the analysis on the most commonly published and applied AAs since not all $\delta^{15}N$ values are reported in all studies. AAs were a priori classified as either trophic [Glu, alanine (Ala), leucine (Leu), isoleucine (Ile), aspartic acid (Asp), valine (Val), proline (Pro)] or source AAs [Phe, lysine (Lys), glycine (Gly), serine (Ser), histidine (His), threonine (Thr) and tyrosine (Tyr)] based on previous findings (McClelland and Montoya 2002; Popp et al. 2007).

To assess the applicability of TP estimation across TLs, we correlated $\delta^{15}N_{Glu}$ values with $\delta^{15}N_{Phe}$ values for each $TP_{literature}$ in intervals of 0.5 TP using a type II regression (Warton et al. 2006), and fitted $\delta^{15}N_{Glu} - \delta^{15}N_{Phe}$ values in relation to $TP_{literature}$ for all individual species. Similarly, we fitted $\delta^{15}N$ values from other individual trophic AAs relative to $\delta^{15}N_{Phe}$ values to test which combinations of AA $\delta^{15}N$ values provide consistent TP estimates. Individual TEFs were calculated based on the $TP_{literature}$ estimates by rearranging Eq. 1 and averaged across feeding ecology and N excretion. For each species, an individual TEF was calculated for $\delta^{15}N$ values of each trophic AA (Glu, Ala, Leu, Ile, Asp, Val, Pro) always with $\delta^{15}N_{Phe}$ values as the source AA. In all analyses β-values were calculated based on available primary producer data (Chikaraishi et al. 2007, 2009, 2014; Maeda et al. 2012; McCarthy et al. 2007, 2013; McClelland et al. 2003; McClelland and Montoya 2002). The effect of feeding ecology (herbivore, omnivore, carnivore) and form of N excretion (CH_4N_2O, NH^+_4) on TEF was tested using one-way ANOVA and Tukey honest significant difference post hoc tests for differences among groups. For N excretion analysis only species with TP of three or higher were included in the one-way ANOVA to diminish any TP effect on TEF values since the CH_4N_2O-excreting animals occupied only higher TPs. Organism size (i.e. organism length) was originally included as a random effect in the one-way ANOVA comparisons to account for any influence on AA ^{15}N enrichment due to organisms size or ontogenetic change; however, as size had no significant influence on any ANOVA outcomes, this was not considered further.

TP models using $\delta^{15}N$ values from multiple AAs

We constructed a multiple AA modelling framework that allows researchers to retrieve TP information from isotope values of various AAs that are available or of particular interest. Trophic AAs and source AAs were selected based on correlations between commonly measured trophic AA $\delta^{15}N$ values relative to $\delta^{15}N_{Glu}$ values, and source AA $\delta^{15}N$ values relative to $\delta^{15}N_{Phe}$ values (ESM Appendix C). A slope not significantly different from one in a cross-plot of $\delta^{15}N$ values of a given trophic AA versus $\delta^{15}N_{Glu}$ and for $\delta^{15}N$ values of a given AA source relative to $\delta^{15}N_{Phe}$ values, respectively, implies similar ^{15}N enrichment across measurements. In cases where the slope of these cross-plots was not significantly different from one, the intercept (AA_{diff}) denotes whether a constant offset between the $\delta^{15}N$ values of these two AAs is present. Assessment of slopes was done by fitting type II correlations of the data relative to a slope of one (Warton et al. 2012). The $\delta^{15}N_{Glu}$ and $\delta^{15}N_{Phe}$ values were consistently used as a reference because these AAs were justified by Chikaraishi et al. (2009) as the best compounds to estimate TP and are the most commonly reported $\delta^{15}N$ AA values across species and TLs. By normalizing isotope values of trophic AAs relative to Glu and source AAs relative to Phe, models thus rely only on the $\beta_{Glu\text{-}Phe}$ value and a combined TEF value, calculated from the $TP_{literature}$ estimates (ESM Appendix C). Here, normalization between AA isotope values was done using linear type II regression coefficients based on data from multiple measurements across TLs; however, in principle, normalization could similarly be done using relative difference between isotope values of AAs in primary producers (i.e. β-values, see ESM Appendix A).

For each parameter in the multiple or single AA isotope modelling framework an uncertainty term encompassing instrumental and biological uncertainty was included and computed as the SD from a normal distribution around the mean value of each individual AA $\delta^{15}N$ value, TEF value and the β-coefficient. The performance of each TP estimation model was evaluated for both single AA and multiple AAs models, all constructed based on the general formulation:

$$TP_{x/y} = \left(\frac{\sum(\delta^{15}N_x i \pm \mathrm{SD}\ \delta^{15}N_x i + \delta^{15}N_{\mathrm{diff}} i)/X - \sum(\delta^{15}N_y j \pm \mathrm{SD}\ \delta^{15}N_y j + \delta^{15}N_{\mathrm{diff}} j)/Y - \beta_{x/y} \pm \mathrm{SD}\ \beta_{x/y}}{\Delta_x - \Delta_y \pm \mathrm{SD}\ \Delta_x - \Delta_y} \right) + 1 \quad (2)$$

where, $N_x i$ are the $\delta^{15}N$ values of trophic AA_i, and $N_y j$ are the $\delta^{15}N$ values of source AA_j. The correction term ($N_{diff} i$) was applied to normalize $\delta^{15}N$ values of a given AA trophic relative to $\delta^{15}N_{Glu}$ values, and similarly $N_{diff} j$ to normalize $\delta^{15}N$ values of a given source AA relative to $\delta^{15}N_{Phe}$ values (normalization was only relevant when the compiled metadata were modelled). X and Y denote the number of trophic AA(s) and source AA(s) $\delta^{15}N$ values included in a given model, and Δ_x and Δ_y are the ^{15}N enrichment factors with each trophic transfer for AA(s) x and y, respectively (i.e. the TEF_{x-y}). $\beta_{x/y}$ is the difference between the $\delta^{15}N$ values of trophic AA(s) x and source AA(s) y in primary producers. The SD term for each parameter represents the uncertainty. For all modelling, except if stated otherwise, an average TEF of 5.9 ‰, calculated from all trophic AAs relative to Phe using the $TP_{literature}$ estimate and Eq. 1 was applied (ESM Appendix C). $\beta_{Glu\text{-}Phe}$ derived from the primary producer metadata was set to 2.9 ‰ ($n = 47$) for all models. Model scenarios were run using a bootstrapping approach with 5000 simulations. Additional information on the model framework is available in ESM Appendix C.

First, we tested model performance with inclusion of several trophic and source AAs using fictive $\delta^{15}N$ values and evaluated output in terms of SD of the TP estimate. Using a SD of 1 for all $\delta^{15}N$ AA values, TEF and β model performance was assessed for all combinations of one to six AA trophic(s) and/or AA source(s). To cover a realistic range from two to four TP, all $\delta^{15}N$ source AA values were fictively assumed to be zero, while all $\delta^{15}N$ trophic AA values were fictively assumed to be the same value: either nine, 15, or 21. Based on a realistic range of previously applied values, we also tested the influence of varying TEF (4.4, 5.9 and 7.6 ‰) on TP model uncertainty, with differences in $\delta^{15}N$ AA values in this case adjusted to always represent a species with a TP ~ 3 to allow comparison of the SD model output.

Second, we modelled TP using the metadata of individual $\delta^{15}N$ AA values. Model outputs were fitted against $TP_{literature}$ and the SD of the TP output and the corrected Akaike information criterion (AICc) reported. TP model simulations were done using $\delta^{15}N$ AA values from all combinations of one to six trophic AA(s) and/or one to two source AA(s). For each trophic AA an individual SD was calculated based on the residual values from the regression between that individual AA $\delta^{15}N$ value and the mean of all trophic $\delta^{15}N$ AA values. Since only two source AAs were included, calculation of SD based on the residuals of $\delta^{15}N$ AA values was not possible and instead a conservative fixed SD of 1 was used, which was not sensitive to model outcomes. SD for TEF (SD = 1.7) and $\beta_{Glu\text{-}Phe}$ (0.9) was used from measured values (Chikaraishi et al. 2009). $\beta_{Glu\text{-}Phe}$ (2.9 ‰) could be used in all TP models of the metadata since all trophic AAs were normalized relative to Glu and the source AAs to Phe using the $AA_{diff} i$ and $AA_{diff} j$ values. TEF values for each model

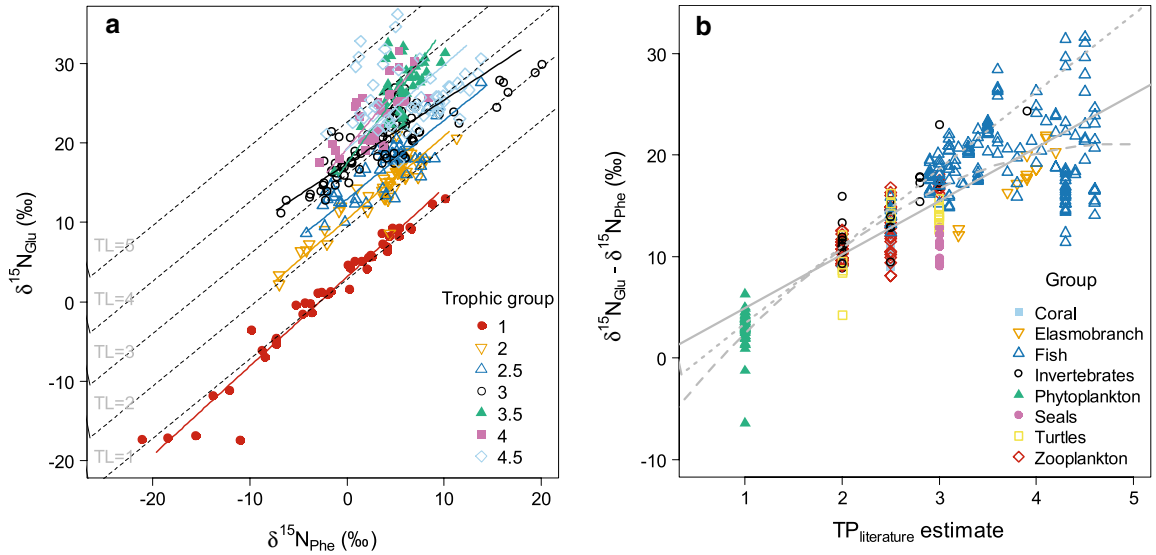

Fig. 1 $\delta^{15}N$ values of glutamic acid (*Glu*) and phenylalanine (*Phe*) measured across different trophic levels (TL) and related to literature trophic position ($TP_{literature}$) estimation. **a** $\delta^{15}N_{Glu}$ and $\delta^{15}N_{Phe}$ values, grouped in 0.5-TP intervals based on $TP_{literature}$ estimates. *Coloured lines* denote slope between $\delta^{15}N_{Glu}$ and $\delta^{15}N_{Phe}$ values for each TL (n = 26–86). *Dotted grey lines* indicate fictive TL (1–5) position calculated using a trophic enrichment factor $(TEF)_{Glu\text{-}Phe}$ of 6.6 ‰ and $\beta_{Glu\text{-}Phe}$ of 2.9 ‰. **b** $\delta^{15}N_{Glu}$ and $\delta^{15}N_{Phe}$ values in relation to $TP_{literature}$ estimates for major animal groups. *Solid grey line* indicates type II fitted regression (r^2 = 0.70, n = 347, $p < 0.01$, slope = 6.3). *Dashed grey line* denotes an exponential fit indicative of non-constant TEF (r^2 = 0.75, n = 347, $p < 0.01$). *Dotted grey line* indicates linear TL prediction proposed by Chikaraishi et al. (2009) (colour figure online)

combination are presented in ESM Appendix C. To exclude unrealistic TP values from the model output, models were posteriorly adjusted by conservatively excluding values ±2 from each species' model-estimated mean TP. All modelling and statistical analyses were performed using the R software environment 2.14.1 (R Development Core Team 2014).

Results

Analysis of the compiled data set showed that $\delta^{15}N_{Glu}$ and $\delta^{15}N_{Phe}$ values are linearly correlated across TP supporting the general application of AA-CSIA regardless of absolute $\delta^{15}N$ AA values (Fig. 1a). $\delta^{15}N_{Glu}$ and $\delta^{15}N_{Phe}$ values for lower trophic groups (TP one to three) were highly correlated ($r^2 > 0.69$, $p < 0.01$, df = 44–85), whereas variation increased at higher TP (TP 3.5, r^2 = 0.39, $p < 0.01$, df = 39; TP 4: r^2 = 0.48, $p < 0.01$, df = 25; TP 4.5: r^2 = 0.01, p = 0.49, df = 49). The difference between $\delta^{15}N_{Glu}$ and $\delta^{15}N_{Phe}$ values plotted as a function of $TP_{literature}$ for all individual taxa showed a strong positive correlation (r^2 = 0.70, df = 346, $p < 0.01$) with a regression slope of 6.3 ± 0.35 (95 % confidence interval) (Fig. 1b), which relates to $TEF_{Glu\text{-}Phe}$. A common slope test showed that the regression slope of 6.3 ± 0.35 derived from the metadata was significantly lower than a slope based on a $TEF_{Glu\text{-}Phe}$ value of 7.6 ‰ as proposed by Chikaraishi et al. (2009) ($p < 0.01$, df = 346). The difference between slopes was not significant when comparing lower TL species (TL ≤ 3, p = 0.44, df = 227). An exponential model provided a slightly better fit to the metadata (r^2 = 0.75, df = 346, $p < 0.01$); however, this fit was driven by few fish measurements (*Thunnus albacares*) (Lorrain et al. 2014; Olson et al. 2010) with high estimated $TP_{literature}$ values. Excluding those measurements, the linear (r^2 = 0.82) and exponential model (r^2 = 0.83) performed equally well. $\delta^{15}N$ values of other trophic AAs relative to $\delta^{15}N_{Phe}$ values also correlated strongly with $TP_{literature}$ (ESM Appendix B).

TEF values for each trophic AA relative to Phe were calculated by rearranging Eq. 1. For the commonly used $TEF_{Glu\text{-}Phe}$ the average value across all compiled data was 6.6 ‰ ± 1.7 SD (ESM Appendix C; Fig. 2). The $TEF_{Glu\text{-}Phe}$ (6.6 ± 1.7 ‰) calculated by rearranging Eq. 1 for each individual species (ESM Appendix C) differed slightly from the proposed $TEF_{Glu\text{-}Phe}$ (6.3 ± 0.35 CI) based on the type II regression analysis (Fig. 1b). Please note that we focus on the TEF values calculated by rearranging Eq. 1 and consistently use these values for all subsequent modelling and comparisons between feeding types.

TEF values based on the N isotopic composition of commonly applied trophic AAs and the source AA Phe varied with feeding ecology. $TEF_{Glu\text{-}Phe}$, $TEF_{Ala\text{-}Phe}$, $TEF_{Asp\text{-}Phe}$, $TEF_{Val\text{-}Phe}$ and $TEF_{Pro\text{-}Phe}$ values were significantly lower for carnivores and omnivorous compared to herbivores (Fig. 2), except for $TEF_{Pro\text{-}Phe}$ in the latter

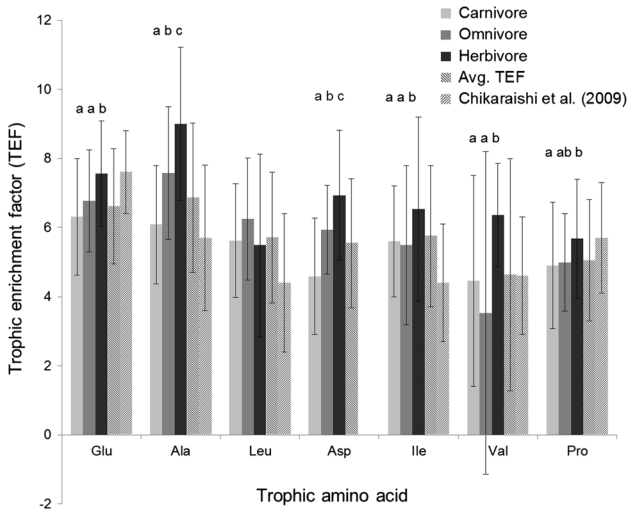

Fig. 2 Average TEF by feeding type calculated from Eq. 1 using $TP_{literature}$, β for trophic amino acid (AA) $\delta^{15}N$ values [Glu, alanine (*Ala*), leucine (*Leu*), isoleucine (*Ile*), valine (*Val*), proline (*Pro*), aspartic acid (*Asp*)]) relative to $\delta^{15}N_{Phe}$ values. *Light grey* Carnivore $TEF_{Trophic\ AA\text{-}Phe}$ (n = 56–197), *grey* omnivore $TEF_{Trophic\ AA\text{-}Phe}$ (n = 38–56), *dark grey* herbivore$_{Trophic\ AA\text{-}Phe}$ (n = 28–41), *hatched grey* average $TEF_{Trophic\ AA\text{-}Phe}$ (n = 122–294) combined for all feeding types in the metadata, *hatched light grey* average $TEF_{Trophic\ AA\text{-}Phe}$ from Chikaraishi et al. (2009). Test statistics (*a*, *b*, *c*) denote significant differences between feeding groups for individual AAs, respectively, based on ANOVA–Tukey post hoc test. For other abbreviations, see Fig. 1

case. $TEF_{Ala\text{-}Phe}$ and $TEF_{Asp\text{-}Phe}$ were also significantly lower for carnivores compared to omnivores. The ^{15}N enrichment of several trophic AAs relative to Phe also varied between organisms excreting either CH_4N_2O or NH^+_4. Values of $TEF_{Glu\text{-}Phe}$, $TEF_{Ala\text{-}Phe}$, $TEF_{Ile\text{-}Phe}$, $TEF_{Leu\text{-}Phe}$, $TEF_{Asp\text{-}Phe}$ and $TEF_{Val\text{-}Phe}$ were lower for CH_4N_2O-excreting animals compared to NH^+_4 (one-way ANOVA, df = 83–216, F = 11.71–166.40, $p < 0.01$; ESM Appendix Fig. D1). $TEF_{Pro\text{-}Phe}$ showed no difference (one-way ANOVA, df = 157, F = 0.30, p = 0.59) between types of N excretion.

Modelling TP using $\delta^{15}N$ values from multiple AAs

Simulation models with fictive $\delta^{15}N$ AA values were done for all combinations of one to six trophic and/or source AAs, with 1 SD for all $\delta^{15}N$ AA values, TEF and β, covering a range of TP ~ 2–4 (Fig. 3a–c). Model uncertainty decreased consistently with each stepwise inclusion of $\delta^{15}N$ values from additional AAs as indicated by the decline in SD of TP estimation, particularly after the inclusion of $\delta^{15}N$ values from two or more trophic and/or source AAs (Fig. 3a–c). Increased uncertainty was present for higher TP; still models including more than two AAs performed better (Fig. 3c). TP model uncertainty was further dependent on the TEF value and model performance improved with increasing TEF values (Fig. 3d). The change in magnitude of the TP SD was most sensitive to the TEF value, while an increased SD of $\delta^{15}N$ AA values and β had less

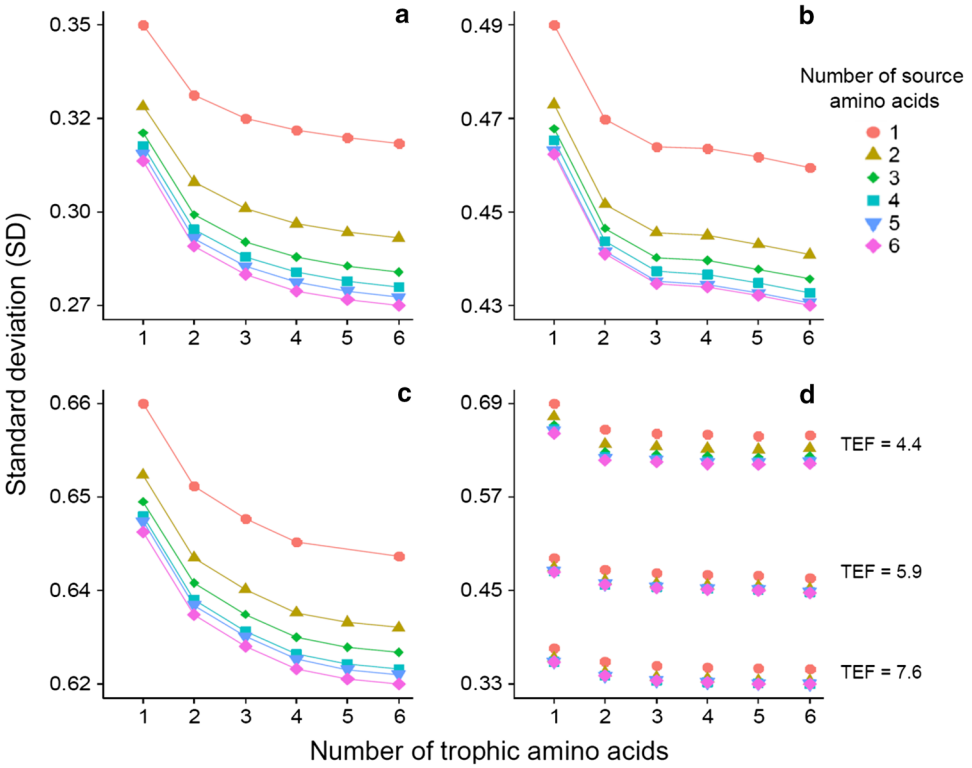

Fig. 3 Fictive model uncertainty output plotted as the SD of the TP estimate. Output from all model combinations using one to six trophic and/or source $\delta^{15}N$ AA values, with 1 SD used for all parameters (TEF, β, $\delta^{15}N$ AA values). β applied was 2.9 ‰ in all figures. SD of model output for **a** fictive $\delta^{15}N$ AA values representing TP = 2, **b** TP = 3 and **c** TP = 4. Number of trophic AAs is denoted on the *x-axis* and number of source AAs is shown by *different coloured symbols*. **d** SD of TP model output for simulations with varying TEF (4.4 ‰, 5.9 ‰, 7.6 ‰) for $\delta^{15}N$ values of AA source(s) and AA trophic(s) adjusted to represent TP = 3 in all cases. Number of trophic AA(s) is denoted on the *x*-axis and *symbols* denote number of source AAs. Note that *y*-axes have different scales

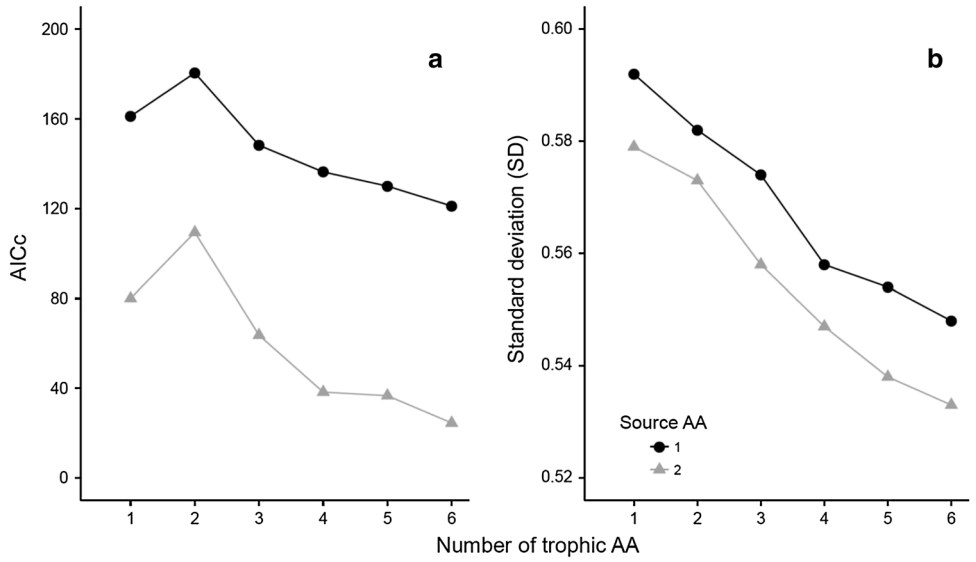

Fig. 4 Evaluation of model combinations based on the metadata $\delta^{15}N$ AA values ($n = 108$). **a** Corrected Akaike information criterion (*AICc*) scores and **b** TP model SD, of all TP model combinations. AICc scores were calculated by comparing TP model outputs to $TP_{literature}$ estimates. *Black circles* denote models using one AA source ($\delta^{15}N_{Phe}$ values), *grey triangles* denote models using two source AAs ($\delta^{15}N_{Phe}$ and $\delta^{15}N_{Lys}$ values). TEF values for each model combination are presented in ESM Appendix C and β was 2.9 ‰. The one trophic AA-one source AA model represents the commonly applied model with $\delta^{15}N_{Glu}$ and $\delta^{15}N_{Phe}$ values as proposed by Chikaraishi et al. (2009). Trophic AAs were added stepwise in the following order: Glu, Ala, Leu, Ile, Asp and Pro (the order of AAs did not significantly change the relative difference between model performance). For other abbreviations, see Fig. 1

influence on the uncertainty in TP estimation (data not shown). For high TPs, sensitivity of SD of the TEF was almost twice as high compared to the SD of $\delta^{15}N$ AA values and β.

Based on our metadata, $\delta^{15}N$ values from six trophic AAs (Glu, Ala, Leu, Ile, Asp and Pro) and two source AAs (Phe, Lys) were suitable for inclusion in the multiple model framework as suggested by slope estimation (i.e. a slope not significantly different from one in a cross-plot of $\delta^{15}N$ values of a given trophic AA versus $\delta^{15}N_{Glu}$ or for $\delta^{15}N$ values of a given AA source relative to $\delta^{15}N_{Phe}$ values). In fact, $\delta^{15}N$ AA values from all six trophic AAs had very high correlations ($r^2 > 0.91$), showing very similar ^{15}N enrichment across TLs. Similar, significant positive correlations were observed between $\delta^{15}N$ AA values of the source AAs Lys, Tyr, His and Phe ($r^2 > 0.74$) (ESM Appendix Table C). However, only Phe and Lys were used for the multiple AA modelling of the metadata since too few $\delta^{15}N$ AA values were present from His ($n = 15$) and Tyr ($n = 50$).

Model performance of the metadata with individual $\delta^{15}N$ AA values and SDs, respectively, improved when more trophic AAs were included (Fig. 4a, b). The best model fit in terms of TP SD and AICc relative to $TP_{literature}$ was the highest multiple model including six trophic AAs and two source AAs (AICc = 24.5, $n = 108$) (Fig. 4b). Including two rather than one source AA with similar SD improved the TP modelling and even the one trophic AA-two source AA model (AICc = 80.0, $n = 108$) performed better than a six trophic AA-one source AA model (AICc = 121.1, $n = 108$). The higher SD associated with the $\delta^{15}N_{Ala}$ values (the second trophic AA included) resulted in models of one trophic AA (only $\delta^{15}N_{Glu}$ values) with one to two source AAs to produce a more accurate TP estimate than models including two trophic AAs ($\delta^{15}N_{Glu}$ and $\delta^{15}N_{Ala}$ values) with one to two source AAs. However, the influence of higher SD from single $\delta^{15}N$ AA values diminished with inclusion of $\delta^{15}N$ values from more than two trophic AAs.

Discussion

Our meta-analysis confirmed the applicability of using AA $\delta^{15}N$ values as a broad-scale tool to predict the TP of multiple marine organisms at all TLs (Fig. 1a), in agreement with previous studies (Chikaraishi et al. 2009, 2014; Hannides et al. 2009; McClelland and Montoya 2002; Schmidt et al. 2004). For the commonly used $TEF_{Glu\text{-}Phe}$ value our metadata indicated a value of 6.6 ± 1.7 ‰ across all taxa (ESM Appendix C; Fig. 2), which may be further influenced by feeding type and the form of N excretion. Our results also showed that both the absolute TEF and uncertainty in the TEF value were the most influential sources of error for TP estimation, and that more precise TP estimates can, in principle, be obtained by including multiple trophic and source $\delta^{15}N$ AA values from an individual organism.

$\delta^{15}N$ values of trophic and source AAs

The $\delta^{15}N$ values of six trophic AAs all increased consistently with TP. However, the magnitude of change in the $\delta^{15}N$ values of these trophic AAs relative to that of $\delta^{15}N_{Phe}$ varied. This variability of $\delta^{15}N$ across trophic AAs suggests that ^{15}N enrichment in trophic AAs relative to source AAs may not be constant, though increased inaccuracy of $TP_{literature}$ values of higher TL species may also influence this pattern. Variation was present both between and within taxa, which is in concurrence with previous observations (Choy et al. 2012; McCarthy et al. 2013). The variable ^{15}N enrichment is likely not directly linked to TP because the linear and exponential model fits performed equally well, a pattern also visible for the $\delta^{15}N$ values of other trophic AAs relative to Phe (ESM Appendix B). Bradley et al. (in review) have previously established for a larger number of teleosts that ^{15}N enrichment for several trophic AAs also seems to be linear with TP, similar to original conclusions in Chikaraishi et al. (2009). Thus, variation in ^{15}N enrichment between diet and consumer (i.e. for both $\delta^{15}N_{Glu}$ values and $\delta^{15}N$ values from other trophic AAs relative to $\delta^{15}N_{Phe}$ values) is likely not just explained by increasing TP.

A caveat of our analysis is that we scaled TP estimation based on AA-CSIA with $TP_{literature}$ estimates. $TP_{literature}$ estimates based on conventional stomach or gut content analysis contain known uncertainties as this method provides primarily a snapshot of recently consumed dietary items and may also be susceptible to bias depending on the degradation time of the various dietary components in the gut (Baker et al. 2014; Hynes 1950; Hyslop 1980; Rindorf and Lewy 2004). Nonetheless, $TP_{literature}$ values were considered the best and only standardized method for elucidating any consistent patterns across all species $\delta^{15}N$ AA values in natural samples. However, for several species included in our analysis uncertainties from $TP_{literature}$ estimates may be at least 0.5 TP (Froese and Pauly 2012) and thus comparable to AA-CSIA TP estimates. AA-CSIA methods are likely more precise when reliable species' dietary information is difficult or impossible to retrieve, or from organisms in complex feeding guilds, as is the case for many omnivorous species.

A key benefit of applying AA-CSIA for TP estimation is that consumer source $\delta^{15}N$ AA values retain information from the producer that synthesized them. This was consistently the fact for $\delta^{15}N_{Phe}$ and $\delta^{15}N_{Lys}$ values, which changed little across TLs. Similarly, $\delta^{15}N_{His}$ and $\delta^{15}N_{Tyr}$ values changed little with each trophic transfer and thus are suitable candidates for source AAs; unfortunately $\delta^{15}N_{His}$ and $\delta^{15}N_{Tyr}$ values are not routinely analysed as they typically appear low in concentration or are difficult to analyse (Chikaraishi et al. 2009; McCarthy et al. 2013). $\delta^{15}N_{Ser}$ and $\delta^{15}N_{Gly}$ values showed little promise as source AAs as their $\delta^{15}N$ values increased sometimes substantially with each trophic transfer, a pattern also noted by Hoen et al. (2014). Interestingly, $\delta^{15}N_{Thr}$ values decreased rather systematically with each trophic transfer. The mechanism for this consistent decrease in $\delta^{15}N_{Thr}$ values with TL is unknown; nevertheless recent observations (Bradley et al., in review) have led to suggestions that $\delta^{15}N_{Thr}$ values in combination with certain trophic $\delta^{15}N$ AA values may yield TP information. Results of this meta-analysis indicate that, so far, consistent source AA information is mostly limited to $\delta^{15}N_{Phe}$ and $\delta^{15}N_{Lys}$ values.

Modelling TP using multiple $\delta^{15}N$ AA values

Our resampling model approach showed that including $\delta^{15}N$ values from more than one trophic and one source AA can improve precision in TP estimation (Fig. 3a–c). Sound TP estimation relies on both accuracy (i.e. under- or overestimation of TP) and precision (i.e. the uncertainty of TP estimate) and we suggest critical evaluation of both before applying any set of AAs for estimating TP. Here, improvement of model precision (i.e. a decrease in the SD of the TP estimation) was especially noticeable when including $\delta^{15}N$ values of at least two trophic AAs and two source AAs (Fig. 3a–c), even when each AA had different uncertainty values (e.g. uncertainty based on the SD of residual values was higher for $\delta^{15}N_{Ala}$ than $\delta^{15}N_{Glu}$ values; Fig. 4a, b). Thus, even if $\delta^{15}N_{Glu}$ and $\delta^{15}N_{Phe}$ values provide the most precise single AA model estimate (Chikaraishi et al. 2009), incorporating additional AA $\delta^{15}N$ values may result in improved trophic information. However, multiple models may not always be the most precise approach, especially if analytical uncertainty is highly variable between AAs. Thus, researchers should determine and evaluate the uncertainty of the isotope measurements of each individual AA through multiple measurements, as these can vary greatly depending on the abundance of any compound and the ability to separate compounds chromatographically (Hayes et al. 1990). For example, when analysing AAs as N-acetyl-n-propyl AA esters of Lys, His and Tyr can elute too closely, though accurate measures are possible (McClelland and Montoya 2002). When most precise TP estimates are needed, derivatisation and chromatographic techniques should be considered that successfully separate isotope values of AA such as Glu, Ala, Leu, Ile, Asp, Pro, Lys and Phe.

Our modelling additionally showed that both the precision and accuracy of the TEF value is the most influential source for errors in TP estimation (Fig. 3d). Applying inaccurate TEF values can result in TP estimation that may deviate by as much as one TP relative to expectations based on conventional stomach or gut content analysis (Dale et al. 2011). For an organism occupying a high trophic niche in the food web, altering

the absolute $TEF_{Glu\text{-}Phe}$ value from only 7.6 to 6.6 ‰ results in a change in estimated TP of approximately 0.5. Regardless of model type, applying an incorrect TEF value will therefore result in an inaccurate TP. Studies measuring TEF values between diet and consumer should remain a future focus as this is a key component for accurate TP estimation.

Factors influencing TEF and TP estimation

We observed a mean $TEF_{Glu\text{-}Phe}$ value of 6.6 ± 1.7 ‰ for all species in the metadata (ESM Appendix C; Fig. 2). This suggests that the $TEF_{Glu\text{-}Phe}$ may be lower than the commonly applied value of 7.6 ‰, which is in line with previous field observations of fish and zooplankton (Choy et al. 2012; Décima et al. 2013) and controlled feeding studies (Bradley et al. 2014; Hoen et al. 2014). Inaccurate TP estimation seems to concern mostly higher TL species (Dale et al. 2011; McCarthy et al. 2013), although this has also been reported for zooplankton (Décima et al. 2013). And interestingly, TEF values approximating zero for most trophic AAs (except Ala) indicate that AA-CSIA may not fully capture the trophic role of heterotrophic protozoans in pelagic food webs (Gutiérrez-Rodrıguez et al. 2014). In ecosystems where protozoan grazing is substantial, the energy flux through the microbial food web may be unaccounted for and hence a large portion of the ocean's variability in food web structure and function may be overlooked. Bradley et al. (in review) estimated a $TEF_{Glu\text{-}Phe}$ of ~6.0 ‰ for a large number of teleosts, while studies on elasmobranchs (Dale et al. 2011), marine mammals (Germain et al. 2013) and mussels (Vokhshoori and McCarthy 2014) suggest even lower $TEF_{Glu\text{-}Phe}$ values (~3–5 ‰). Although results of our analyses are inconclusive as TEF calculations were based on $TP_{literature}$ estimates with known uncertainties, employing potential taxa-specific TEF values may be necessary to increase the accuracy of TP calculation based on AA-CSIA.

TEF values for trophic AAs relative to Phe even varied considerably between organisms with different feeding ecology. Most TEF values were predominantly higher for herbivores compared to omnivores and carnivores, a pattern in agreement with recent controlled laboratory studies on larger fish (Hoen et al. 2014) and tilapias fed with different diets (Bloomfield et al. 2011). $TP_{literature}$ estimates from omnivorous species, which often feed on a complex mixture across TLs, can also be highly uncertain, and results of our TEF values for this group should be considered with caution. Nonetheless, Bloomfield et al. (2011) showed that for tilapia fed with a plant diet, most trophic AAs (Glu, Ala, Asp, Ile, Leu, Val and Pro) had higher diet to consumer ^{15}N AA enrichment than fish fed with an animal diet, though AA supplementation from symbiotic gut microbes (Arthur et al. 2014; Newsome et al. 2011) may also influence AA isotope composition. However, all $\delta^{15}N$ AA values may not have reached steady-state isotopic composition with their diet given the considerably long incorporation time of certain AA $\delta^{15}N$ values in fish tissue (Bradley et al. 2014). Similar, high ^{13}C enrichment has been observed for non-essential AAs in fish fed plant diets with low amounts of protein (McMahon et al. 2010), and dietary protein composition has similarly been noted to affect consumer bulk ^{15}N enrichment (Gaye-Siessegger et al. 2004; Martínez del Rio and Wolf 2005; Vanderklift and Ponsard 2003). These results support our findings that for several trophic AAs the ^{15}N enrichment seems to be influenced by the balance between the nutritional requirement of the consumer and the diet. Researchers wishing to incorporate TEF values specific to herbivores, omnivores and/or carnivores (e.g. in our analysis, $TEF_{Glu\text{-}Phe}$ estimated to 7.6, 6.8 and 6.3 ‰, respectively; ESM Appendix C) or form of N excretion may consider application of a scaled TP estimation as a way to improve TP estimation (Germain et al. 2013; Hoen et al. 2014).

For all trophic AAs our analysis showed TEF values lower than 5 ‰ for CH_4N_2O-excreting animals (i.e. seals, penguins, elasmobranchs) compared to animals excreting NH^+_4 ($TEF_{Trophic\ AA\text{-}Phe}$ values: 4.85–6.49 ‰) (i.e. fish, invertebrates). Here, analysis was done comparing $\delta^{15}N$ AA values from both blood and muscle tissues and thus should be taken with some caution. However, previous studies on seals have noted only minor difference in most $\delta^{15}N$ AA values between blood and muscle tissues (Germain et al. 2013). Studies on CH_4N_2O-excreting penguins (Lorrain et al. 2009), seals (Germain et al. 2013) and elasmobranchs (Dale et al. 2011) have suggested this pattern reflects differences in N metabolism, though, Hoen et al. (2014) noted no such differences in TEF between three species of sharks and a carnivorous fish held in captivity.

Other factors than the TEF may also influence consumer $\delta^{15}N$ AA values. Species migrating between habitats will, for a considerable time, retain the dietary $\delta^{15}N$ AA values from previous foraging areas (Madigan et al. 2014). And interestingly, Bradley et al. (2014) showed for Pacific blue fin tuna (*Thunnus orientalis*) undergoing a diet shift in captivity that individual AA isotopic incorporation times vary greatly, with individual AA half-lives spanning 28–305 days. Similar variability in individual AA ^{15}N incorporation times have also been observed in Pacific white shrimp (*Litopenaeus vannamei*) (Downs et al. 2014). This means that for species shifting dietary sources any mismatch in isotopic incorporation rates between trophic and source AAs should be understood to avoid potential errors in TP estimation. The N AA isotope incorporation into different consumer tissues may also vary with both tissue composition and organism physiology (Schmidt et al. 2004). And even if ^{15}N enrichment is similar between

different body tissues (Chikaraishi et al. 2014; Germain et al. 2013), temporal variation in isotope incorporation still differs substantially (Dalerum and Angerbjorn 2005; Trueman et al. 2012), and for structures such as scales or otoliths the N isotope composition is embedded and fixed as growth rings which are continuously layered (Trueman et al. 2012). Trophic information based on δ^{15}N AA values in scales or otoliths thus provides an integrated TP measure, which may differ substantially from that of muscle tissue which infers information about the recent time prior to sampling. Differences in AA incorporation rates encoded in the δ^{15}N AA values, however, also provide unique tracer information at different temporal scales, such as tracing age-specific migration patterns of Pacific blue fin tuna across the Pacific Ocean (Madigan et al. 2014). And increasing insight into N AA isotope incorporation both between and within tissues will likely open up new possibilities for AA-CSIA applications.

Accurate assessment of primary producer δ^{15}N values is another key component when calculating the TP of a consumer. Based on the metadata we calculated a $\beta_{\text{Glu-Phe}}$ of 2.9 ‰, slightly lower than the 3.4 ‰ reported by Chikaraishi et al. (2009) with no consistent differences between $\delta^{15}N_{Glu}$ and $\delta^{15}N_{Phe}$ values among marine phytoplankton species, though differences have been observed for combinations of other δ^{15}N AA values (McCarthy et al. 2013). However, large differences between δ^{15}N values of primary producers, including $\beta_{\text{Glu-Phe}}$, exist between terrestrial and marine plants (Chikaraishi et al. 2010). Therefore, a priori knowledge on the potential dietary source pool is still important, especially for marine coastal species. Vander Zanden et al. (2013) showed that seagrasses (*Thallasia testudium*) have $\beta_{\text{Glu-Phe}}$ of −8.4 ‰ consistent with the evolution of seagrasses from terrestrial C3 plants. Thus, to obtain realistic TP estimates for marine green sea turtles (*Chelonia mydas*) known to forage on seagrass, Vander Zanden et al. (2013) applied a $\beta_{\text{Glu-Phe}}$ of −8.4 ‰. In fact, differences in some δ^{15}N AA values between terrestrial and marine sources can be highly informative by providing powerful tracers in complex ecosystems to evaluate the relative significance of allochthonous and autochthonous inputs to the nutrient pool in aquatic ecosystems (Ishikawa et al. 2014).

Future directions

To advance the already strong application of AA-CSIA we suggest that it is critical that future research focus on factors influencing the value of TEF. Both the absolute value of TEF and the SD of TEF were found to be the most influential parameters in obtaining accurate TP estimates. $\delta^{15}N_{Phe}$ and $\delta^{15}N_{Lys}$ values were found to be reliable source AAs across species, while increased knowledge on $\delta^{15}N_{His}$ and $\delta^{15}N_{Tyr}$ values is encouraged as these source AAs may be capable of providing further insight into consumer dietary incorporation. Full understanding of the influence of nutritional quality, form of excretion and differences in isotopic incorporation rates of individual AAs and between tissues, on ^{15}N enrichment should be explored in detail, as this will broaden the use of AA-CSIA. Nevertheless, TP estimation from AA-CSIA provides a sound understanding of trophic structures, which is likely more accurate or comparable to conventional methods based on dietary assessment, especially when reliable species' dietary information is difficult to retrieve. Thus, depending on research aims, we encourage application of AA-CSIA in combination with complimentary diet assessment to gain further detailed insight into trophic structures and energy pathways.

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