

Trophic discrimination of nitrogen stable isotopes in amino acids varies with diet quality in a marine fish

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Abstract

Analysis of individual amino acid (AA) $\delta^{15}\text{N}$ values is increasingly common in studies of food web architecture, movement ecology, and biogeochemical cycling. However, observations that nitrogen isotope fractionation of AAs may vary across species and trophic positions (TP) complicate the application of compound-specific stable isotope analysis (CSIA) in studies of aquatic food webs. We reared a common estuarine fish (*Fundulus heteroclitus*) on four diets to test the hypothesis that diet composition significantly affects AA trophic discrimination factors (TDFs) derived from normalizing fractionation of trophic AAs to the canonical source AA phenylalanine. Phenylalanine showed little trophic fractionation regardless of diet composition, confirming its use as a proxy for $\delta^{15}\text{N}$ values at the base of food webs. However, TDF values showed significant negative relationships with two important measures of diet quality, protein content and AA imbalance between diet and consumer. As a result, consumers feeding on high protein diets with AA compositions matching their own had significantly lower TDF values than previous literature values, while consumers feeding on contrasting low quality diets had significantly higher TDF values than previously reported. Our results provide an explanation for the apparent relationship between TDF value and TP, and highlight a significant limitation when using a single TDF value among all trophic transfers within a food web. Accurate determination of TPs using CSIA will likely require multiple TDF values across trophic levels or further exploration of other trophic AAs, such as proline, that may show less variation in TDF with diet composition than glutamic acid.

Stable isotope analysis (SIA) has been widely used in ecosystem research since the 1970s to identify patterns of resource utilization, characterize trophic relationships, and reconstruct animal movements (Peterson and Fry 1987; Boecklen et al. 2011; McMahon et al. 2013a,b). The use of bulk tissue stable nitrogen isotope values to link diet and consumer relies upon the assumption that the $\delta^{15}\text{N}$ value of a consumer reflects the weighted mean of the isotopic composition of its diet with some trophic fractionation (DeNiro and Epstein 1981; Minagawa and Wada 1984). This relationship is typically used to calculate a bulk trophic position (TP_{Bulk}) according to the following equation:

$$\text{TP}_{\text{Bulk}} = \lambda + \left[\frac{\delta^{15}\text{N}_{\text{consumer}} - \delta^{15}\text{N}_{\text{baseline}}}{\Delta^{15}\text{N}_{\text{C-D}}} \right] \quad (1)$$

where $\delta^{15}\text{N}_{\text{consumer}}$ and $\delta^{15}\text{N}_{\text{baseline}}$ represent the bulk stable nitrogen isotope values of the consumer and baseline primary producer(s) supporting the food chain, respectively, λ

is the TP_{Bulk} of the baseline end member(s), and $\Delta^{15}\text{N}_{\text{C-D}}$ is the trophic fractionation factor calculated from the difference between the stable isotope value of the consumer ($\delta^{15}\text{N}_{\text{C}}$) and diet ($\delta^{15}\text{N}_{\text{D}}$),

$$\Delta^{15}\text{N}_{\text{C-D}} = \delta^{15}\text{N}_{\text{C}} - \delta^{15}\text{N}_{\text{D}} \quad (2)$$

While the bulk nitrogen SIA approach has been commonly applied in the fields of trophic and nutritional ecology (Boecklen et al. 2011), there are a number of challenges to interpreting bulk $\delta^{15}\text{N}$ data that derive from uncertainty in two key parameters of Eq. 1, $\delta^{15}\text{N}_{\text{baseline}}$ and $\Delta^{15}\text{N}_{\text{C-D}}$. First, many ecosystems are characterized by significant spatiotemporal variation in the taxonomic composition of primary producers at the base of the food web, the isotopically distinct nitrogen sources (N_2 , nitrate, ammonia) that support those primary producers, and the efficiency with which those nitrogen sources are utilized. Together, these factors control what is commonly known as the food web “baseline” $\delta^{15}\text{N}$ value ($\delta^{15}\text{N}_{\text{baseline}}$), which may vary by as much as 10‰ over space and time even in the same region and ecosystem (McMahon et al. 2013a,b). Identifying

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appropriate $\delta^{15}\text{N}_{\text{baseline}}$ values typically requires either extensive sampling and characterization of systems or broad assumptions about resource use that can be problematic, particularly when studying highly mobile animals that migrate across isotopically distinct habitats (McMahon et al. 2013a). Second, the $\Delta^{15}\text{N}_{\text{C-D}}$ parameter, which is commonly assumed to be 3.4‰, can in fact vary widely between approximately -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, the biggest challenge to interpreting stable isotope values in bulk tissue samples, particularly of upper trophic level consumers, involves determining whether changes in a consumer's $\delta^{15}\text{N}$ values are due to variability in the $\delta^{15}\text{N}_{\text{baseline}}$, changes in its TP or variability in $\Delta^{15}\text{N}_{\text{C-D}}$, or some combination of all of these factors (Post 2002).

The SIA of individual amino acids (AAs) has recently become a focus of considerable interest to ecologists because a compound-specific approach may provide accurate estimates of both TP and $\delta^{15}\text{N}_{\text{baseline}}$ (McMahon et al. 2013b). McClelland and Montoya (2002) were the first to demonstrate that the documented increases in bulk $\delta^{15}\text{N}$ values between diet and consumer ($\sim 3.4\text{‰}$) were, in fact, underlain by very different patterns of fractionation among individual AAs. This observation has since been confirmed in multiple systems and species (Chikaraishi et al. 2007, 2009; Germain et al. 2013; Steffan et al. 2013). Individual AAs are, therefore, now commonly divided into two groupings based on their relative fractionation during trophic transfer (Popp et al. 2007). Trophic AAs (e.g., glutamic acid, aspartic acid, alanine, leucine, isoleucine, proline, and valine) undergo significant isotopic fractionation during transamination/deamination, yielding typically large fractionation values ($\Delta^{15}\text{N}_{\text{C-D}} > 5\text{‰}$) compared to bulk $\Delta^{15}\text{N}_{\text{C-D}}$ values. Glutamic acid (Glu), which serves as the main nitrogen shuttle for protein synthesis (e.g., White 2007; McCarthy et al. 2013), is now generally considered to be the best proxy for the entire trophic AA group. Glutamic acid typically has the largest trophic fractionation, is abundant in ecologically relevant tissues, and is well resolved by gas chromatography (e.g., McClelland and Montoya 2002; Chikaraishi et al. 2007, 2009). In contrast, source AAs (e.g., methionine, tyrosine, and phenylalanine) typically show little trophic fractionation between $\delta^{15}\text{N}_{\text{baseline}}$ and measured values in a consumer. Among the source AAs, phenylalanine (Phe) has been found to have the most invariant $\delta^{15}\text{N}$ value during trophic transfer, likely because it does not form or break C–N bonds during metabolic processing (e.g., Chikaraishi et al. 2007, 2009). Therefore, Phe is typically used as the canonical source AA, and its $\delta^{15}\text{N}$ values have been directly applied as a proxy for $\delta^{15}\text{N}_{\text{baseline}}$ in both paleo and modern systems (Lorrain et al. 2009, 2014; Sherwood et al. 2014; Vokhshoori and McCarthy 2014).

The compound-specific stable isotope analysis (CSIA) approach to calculate trophic position (TP_{CSIA}) makes use of characteristic fractionation for trophic and source AAs to provide an internally indexed estimator of TP that is by definition normalized to the $\delta^{15}\text{N}_{\text{baseline}}$. Therefore, unlike TP_{bulk} calculations, the TP_{CSIA} equation does not require explicit knowledge of $\delta^{15}\text{N}_{\text{baseline}}$ as that information is recorded in the consumer source AA $\delta^{15}\text{N}$ values. The most common formulation for TP_{CSIA} is based on the offset between the canonical trophic AA Glu and the canonical source AA Phe:

$$\text{TP}_{\text{CSIA}} = 1 + \left[\frac{\delta^{15}\text{N}_{\text{Glu}} - \delta^{15}\text{N}_{\text{Phe}} - \beta}{\text{TDF}_{\text{Glu-Phe}}} \right] \quad (3)$$

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, β represents the difference in $\delta^{15}\text{N}$ between these same AAs in primary producers, and $\text{TDF}_{\text{Glu-Phe}}$ represents the trophic discrimination factor calculated by normalizing trophic fractionation of Glu ($\Delta^{15}\text{N}_{\text{Glu}}$) to Phe ($\Delta^{15}\text{N}_{\text{Phe}}$),

$$\text{TDF}_{\text{Glu-Phe}} = \Delta^{15}\text{N}_{\text{Glu}} - \Delta^{15}\text{N}_{\text{Phe}} \quad (4)$$

Most applications to date have used Glu and Phe as originally proposed by McClelland and Montoya (2002) and refined by Chikaraishi et al. (2009). However, a number of variations of Eq. 3 have also been proposed to estimate TP_{CSIA} of consumers, differing primarily in the specific trophic and source AAs used (e.g., McClelland and Montoya 2002; McCarthy et al. 2007; Popp et al. 2007; Hannides et al. 2009; Hoen et al. 2014).

While still relatively new, the CSIA approach has been used to examine trophic connections and biogeochemical cycling over a wide range of paleo and modern applications in marine, freshwater, and terrestrial ecosystems (McCarthy et al. 2007; Lorrain et al. 2009; Steffan et al. 2013; Ishikawa et al. 2014; Sherwood et al. 2014). However, perhaps the most problematic assumption underlying the use of CSIA approaches in food web studies is that trophic fractionation for Glu and Phe, and thus $\text{TDF}_{\text{Glu-Phe}}$, remains effectively constant across all consumers and diet types. A $\text{TDF}_{\text{Glu-Phe}}$ value of 7.6‰ was proposed from extensive feeding trials on multiple organisms by Chikaraishi et al. (2007) following nearly identical results from the first rotifer feeding experiments by McClelland and Montoya (2002). This value has been widely adopted by researchers for calculating TP_{CSIA} (Lorrain et al. 2009, 2014; Dale et al. 2011; Choy et al. 2012; Miller et al. 2013; Nakatomi et al. 2014). However, several recent field studies examining TP_{CSIA} of upper trophic level consumers, including penguins, teleost fishes, and elasmobranchs, noted that the use of a $\text{TDF}_{\text{Glu-Phe}}$ value of 7.6‰ led to significantly lower than expected estimates of TP (Lorrain et al. 2009; Dale et al. 2011; Choy et al. 2012). More

recently, several controlled feeding experiments on upper trophic level consumers in marine environments showed definitively that $TDF_{Glu-Phe}$ values vary significantly, and are often substantially lower than 7.6‰ (Germain et al. 2013; Bradley et al. 2014; Hoen et al. 2014). While the $TDF_{Glu-Phe}$ value of 7.6‰ appears robust for lower TP organisms, there is a growing consensus that $TDF_{Glu-Phe}$ values are not constant across all trophic levels. However, the underlying mechanisms generating this variation remain unclear.

To realize the full potential of the CSIA approach, we need to both quantify variability in the $TDF_{Glu-Phe}$ parameter and develop a mechanistic understanding of the underlying biochemical and physiological mechanisms generating variability in $TDF_{Glu-Phe}$ values within food webs. The main objective of this study was, therefore, to test the assumption of constant AA trophic fractionation and thus $TDF_{Glu-Phe}$ values through a controlled feeding experiment. We hypothesized that diet composition is a key determinant of $TDF_{Glu-Phe}$ values in marine food webs. Earlier work based on bulk $\delta^{15}N$ data indicated that the protein content of diets (Roth and Hobson 2000) and the degree of compositional imbalance of individual AAs between diet and consumer can significantly influence bulk $\Delta^{15}N_{C-D}$ (Robbins et al. 2005, 2010; Mill et al. 2007; Florin et al. 2011). These studies found that diets with low protein content and high AA imbalance between diet and consumer led to an increase in bulk N trophic fractionation in consumers. There are a number of potential mechanisms for this pattern, including assimilative and metabolic fractionation, that relate to the degree of AA transamination/deamination during nitrogen metabolism (Gannes et al. 1997; Vander Zanden and Rasmussen 2001). We reared a common marine fish (*Fundulus heteroclitus*, Linnaeus 1766) on diets that varied in protein content and AA composition. We then measured $\delta^{15}N$ values of individual AAs in diet and consumers to examine how trophic fractionation, and thus $TDF_{Glu-Phe}$ value, was influenced by diet composition. This work follows an earlier assessment of carbon isotope fractionation of individual AAs using the same feeding experiment (McMahon et al. 2010), and provides different, but complementary, information about trophic ecology and baseline food web variability.

Methods

Feeding experiment

To examine how diet composition, in the form of protein content and AA composition, affected trophic fractionation of individual AAs, we conducted a controlled feeding experiment with juvenile mummichogs (*F. heteroclitus*) at the Atlantic Ecology Division, United States Environmental Protection Agency in Narragansett, Rhode Island, U.S.A. (McMahon et al. 2010). The experimental design consisted of triplicate tanks with multiple juvenile *F. heteroclitus* reared on one of four compositionally distinct diets: (1) Vegi-Pro,

(2) Bio-Vita, (3) clam, and (4) squid (Table 1). Juvenile *F. heteroclitus*, raised from eggs of wild-caught adult *F. heteroclitus* collected from a salt marsh in Sandwich, Massachusetts U.S.A., were reared in twelve 40 gallon aquaria (four dietary treatments with three tanks per treatment) with flow through seawater at ambient temperature (20°C). Individual aquaria were arranged in two randomly positioned rows under a 12 : 12 h light : dark cycle from fluorescent tubes. Fish were fed to saturation once per day, and tanks were cleaned of excess food and biofilms every three days. Fish were reared on the four diet treatments for eight weeks and more than doubled in biomass during that time. Strong correlations between essential AA $\delta^{13}C$ values in consumers and the new diets ($r^2 = 0.95$) indicated that the fish in the experiment had reached isotopic equilibrium with their diet (McMahon et al. 2010).

The four diets in this study, (1) Vegi-Pro, (2) Bio-Vita, (3) clam, and (4) squid, were selected to represent a range of relative protein content and AA compositions. Vegi-Pro (Freedom Feeds, Urbana, Ohio, U.S.A.) was a plant-based commercial fish pellet that consisted primarily of wheat and soy with a small contribution from corn meal. Bio-Vita (Bio-Oregon, Westbrook, Maine, U.S.A.) was an omnivorous commercial fish pellet that consisted of fish and krill meal, wheat gluten, and whey protein. The two animal-based diets, squid and clam, consisted of freeze-dried and homogenized muscle tissue that we collected from a local fish market in Woods Hole that specializes in locally caught seafood. As such, the time and location of sample collections was well constrained. While for our experimental design TP was an associated variable rather than a central mechanistic driver, TP for each of the diets was calculated directly from their nitrogen CSIA data using Eq. 3 ($n = 3$ aliquots per diet type). Results confirmed expectations for the diet types: Vegi-Pro (plant diet) = 1.0 ± 0.1 , Bio-Vita (omnivorous diet) = 2.0 ± 0.1 , clam (primary consumer diet) = 2.0 ± 0.1 , and squid (secondary consumer diet) = 2.7 ± 0.1 .

Sample preparation and analysis

After eight weeks, all fish were sacrificed in an ice slurry, frozen, and freeze-dried for 72 h. White muscle was removed from each fish, homogenized using a mortar and pestle, and subdivided into portions for bulk tissue SIA and CSIA. For bulk SIA, approximately one milligram of each sample (both fish muscle and diets) was weighed into tin cups and analyzed for $\delta^{15}N$ values using a Europa Hydra 20/20 isotope ratio monitoring-mass spectrometer at the University of California - Davis Stable Isotope Facility, Davis, California, U.S.A. A complete analysis of the bulk tissue results was reported in Elsdon et al. (2010), and only the samples used for CSIA in this study are reported here. Stable isotopes are expressed in standard delta (δ) notation as ‰.

For CSIA, approximately two milligram of sample (both fish muscle and diets) was acid hydrolyzed in 1 ml of 6 N

Table 1. Proximate analysis of crude protein, crude fat, crude fiber, carbohydrate, ash, and moisture content (%) and AA imbalance (difference in AA mol% between diet and consumer) of four diets fed to *F. heteroclitus*: Vegi-Pro, Bio-Vita, clam, and squid (mean \pm SD, $n = 3$).

	Vegi-Pro	Bio-Vita	Clam	Squid	F_{df}
<i>Proximate analysis</i>					
Protein	8.0 \pm 0.4 ^a	53.3 \pm 1.2 ^b	69.1 \pm 0.5 ^c	71.0 \pm 0.4 ^d	$F_{3,8} = 5178^{***}$
Fat	5.9 \pm 0.3 ^a	23.9 \pm 0.8 ^b	17.6 \pm 0.6 ^c	18.0 \pm 0.5 ^c	$F_{3,8} = 479.2^{***}$
Fiber	2.0 \pm 0.2 ^a	0.3 \pm 0.1 ^b	0.3 \pm 0.0 ^b	0.2 \pm 0.0 ^b	$F_{3,8} = 203.6^{***}$
Carbohydrate	72.6 \pm 1.1 ^a	6.0 \pm 0.2 ^b	0.3 \pm 0.1 ^c	0.2 \pm 0.1 ^c	$F_{3,8} = 11608^{***}$
Ash	6.7 \pm 0.3 ^a	10.9 \pm 0.4 ^b	2.9 \pm 0.2 ^c	2.1 \pm 0.1 ^d	$F_{3,8} = 654.2^{***}$
Moisture	6.8 \pm 0.6 ^a	6.0 \pm 0.4 ^a	10.0 \pm 0.8 ^b	8.8 \pm 0.5 ^b	$F_{3,8} = 29.6^{***}$
<i>Amino acid imbalance</i>					
Glutamic acid†	-1.8 \pm 0.2 ^a	-1.0 \pm 0.2 ^b	-0.6 \pm 0.3 ^b	0.2 \pm 0.1 ^c	$F_{3,8} = 40.9^{***}$
Aspartic acid†	-1.7 \pm 0.3 ^a	-1.3 \pm 0.4 ^{ab}	-0.4 \pm 0.2 ^c	-0.8 \pm 0.3 ^{bc}	$F_{3,8} = 10.8^{**}$
Alanine†	-2.4 \pm 0.1 ^a	-1.0 \pm 0.3 ^b	-0.5 \pm 0.2 ^c	0.4 \pm 0.2 ^d	$F_{3,8} = 132.2^{***}$
Isoleucine†	-1.3 \pm 0.1 ^a	-1.3 \pm 0.2 ^a	0.0 \pm 0.1 ^b	0.3 \pm 0.2 ^b	$F_{3,8} = 94.3^{***}$
Leucine†	-1.6 \pm 0.1 ^a	-1.2 \pm 0.3 ^a	0.0 \pm 0.1 ^b	0.8 \pm 0.2 ^c	$F_{3,8} = 98.0^{***}$
Proline†	-0.1 \pm 0.2 ^a	-1.1 \pm 0.1 ^b	0.5 \pm 0.2 ^c	-0.3 \pm 0.1 ^a	$F_{3,8} = 50.8^{***}$
Valine†	-0.3 \pm 0.1 ^a	-0.9 \pm 0.2 ^b	0.9 \pm 0.2 ^c	-0.5 \pm 0.1 ^a	$F_{3,8} = 75.6^{***}$
Glycine#	-2.5 \pm 0.1 ^a	-0.9 \pm 0.3 ^b	-0.7 \pm 0.2 ^b	-0.1 \pm 0.2 ^c	$F_{3,8} = 101.6^{***}$
Serine#	-0.7 \pm 0.1 ^a	-0.7 \pm 0.4 ^a	-0.1 \pm 0.3 ^a	0.5 \pm 0.0 ^b	$F_{3,8} = 16.8^{***}$
Lysine#	-2.8 \pm 0.2 ^a	-3.3 \pm 0.3 ^b	-1.4 \pm 0.2 ^c	-1.2 \pm 0.1 ^c	$F_{3,8} = 124.9^{***}$
Phenylalanine#	-0.9 \pm 0.1 ^a	-0.9 \pm 0.2 ^a	-0.4 \pm 0.1 ^b	-0.5 \pm 0.1 ^b	$F_{3,8} = 15.4^{**}$
Threonine	-1.6 \pm 0.1 ^a	-0.8 \pm 0.2 ^b	-0.1 \pm 0.2 ^c	0.2 \pm 0.2 ^c	$F_{3,8} = 64.9^{***}$

Trophic AAs are indicated by † and source AAs are indicated by #. Different superscript letters indicate significant differences among treatments according to separate one-way ANOVAs with Tukey's HSD post hoc tests, ^{ns} $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

HCl at 110°C for 20 h to isolate the total free AAs. Samples were evaporated to dryness under a gentle stream of N₂. The total free AAs were derivatized by esterification with acidified iso-propanol followed by acylation with trifluoroacetic anhydride (Silfer et al. 1991). The resulting derivatized AAs were brought up in ethyl acetate and injected on column in splitless mode at 250°C and separated on a BPX5 column (60 m \times 0.32 mm inner diameter, 1.0 μ m film thickness; SGE Analytical Science, Austin, Texas, U.S.A.) in a Thermo Trace Ultra gas chromatograph (GC) at the University of California - Santa Cruz, Santa Cruz, California, U.S.A. The separated AA peaks were analyzed on a Finnegan MAT Delta^{plus} XL isotope ratio mass spectrometer interfaced to the GC through a GC-C III combustion furnace (980°C), reduction furnace (650°C), and liquid nitrogen cold trap. The $\delta^{15}\text{N}$ values of twelve individual AAs were analyzed with the previously described method, accounting for approximately 80% of the total hydrolysable AAs. Reconstructed bulk isotope values from mass balanced individual AA $\delta^{15}\text{N}$ values correlated well with bulk SIA values ($r^2 = 0.97$). We classified measured AAs into two groups (Popp et al. 2007): glutamic acid (Glu), aspartic acid (Asp), alanine (Ala), leucine (Leu), isoleucine (Ile), proline (Pro), and valine (Val) were assigned as trophic AAs, while glycine (Gly), serine (Ser), lysine (Lys), and phenylalanine (Phe) were assigned as source AAs. Note that threonine (Thr) $\delta^{15}\text{N}$ values do not behave similarly to either of

these main groups (Hare et al. 1991; McClelland and Montoya 2002; Styring et al. 2010; Germain et al. 2013). Therefore we have listed Thr as a metabolic AA (according to Germain et al. 2013) and do not discuss Thr extensively in this article. Acid hydrolysis converts glutamine (Gln) and asparagine (Asn) into Glu and Asp, respectively, due to cleavage of the terminal amine group, 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. All CSIA samples were analyzed in triplicate along with AA standards of known isotopic composition (Sigma-Aldrich). Standardization of runs was achieved using intermittent pulses of a N₂ reference gas of known isotopic value. Mean reproducibility across all individual AAs was $\pm 0.34\%$. All of the fish and diet samples for CSIA were independent of the samples analyzed for AA $\delta^{13}\text{C}$ values in McMahon et al. (2010).

Data analysis and analytical framework

Following from our experimental design, our data analysis had two main objectives: (1) test the predicted patterns of trophic fractionation for trophic and source AAs, and (2) determine how $\text{TDF}_{\text{AA-Phe}}$ varied as a function of diet composition. We examined the differences in diet composition

(proximate analyses and AA imbalance between diet and consumer) among the four dietary treatments ($n = 3$ aliquots per diet) using separate one-way Analyses of Variance (ANOVAs) and Tukey's Honestly Significant Difference (HSD) post hoc tests ($\alpha = 0.05$). We calculated trophic fractionation factors ($\Delta^{15}\text{N}_{\text{C-D}}$) for bulk SIA and individual AAs by subtracting the mean $\delta^{15}\text{N}$ of the diet ($n = 3$) for each tank from the $\delta^{15}\text{N}$ of individual fish in each tank using Eq. 2. Measurements from individual fish within a tank were not independent, and therefore tanks within treatments ($n = 3$ tanks) were the experimental unit for most subsequent statistical analyses. To test if *a priori* categorizations of individual AAs as trophic and source applied to our experiments, we used separate one-sample *t*-tests to determine if individual AA $\Delta^{15}\text{N}_{\text{C-D}}$ values, averaged across the four diet types, were significantly different from 0‰ ($\alpha = 0.05$). We predicted that, as a group, trophic AA $\Delta^{15}\text{N}_{\text{C-D}}$ would be significantly greater than 0‰ and source AA $\Delta^{15}\text{N}_{\text{C-D}}$ would be close to 0‰. We used separate one-way ANOVAs and Tukey's HSD post hoc tests ($\alpha = 0.05$) to examine differences in individual AA $\Delta^{15}\text{N}_{\text{C-D}}$ values across treatments ($n = 3$ tanks).

For the second objective of the study we investigated the link between diet composition and AA fractionation. Most studies have used the ratio of Glu and Phe for calculating TP_{CSIA} , although several studies have suggested that the mean trophic AA to source AA ratio may provide a more robust estimate of TP (McCarthy et al. 2007; Hannides et al. 2009). We calculated TDF_{CSIA} values for each diet type using all individual trophic source AAs and Phe as the single source AA. We used separate one-way ANOVAs and Tukey's HSD post hoc tests ($\alpha = 0.05$) to examine differences in individual AA $\text{TDF}_{\text{AA-Phe}}$ values across treatments for the trophic AAs, Glu, Asp, Ile, Leu, Pro, and Val ($n = 3$ tanks). We then used linear regression analysis to determine if $\text{TDF}_{\text{AA-Phe}}$ varied as a function of diet composition ($n = 12$ tanks), specifically AA imbalance (difference in trophic AA mol% in diet vs. consumer) and diet total protein content. All statistics were performed in R (ver. 3.0.2).

Results

The diet treatments that we chose for the experiments had significantly different compositions (Table 1). The Vegi-Pro diet had the lowest crude protein (8%) and fat content (6%) and the highest carbohydrate content (73%), while the squid and clam diets had the highest crude protein (69% and 71%, respectively) and fat content (18%) and almost no carbohydrates (Table 1). Bio-Vita protein content was generally intermediate between the Vegi-Pro and the animal-based diets. There were also significant differences in the mol% abundance of AAs between diets and fish muscle across diet types (Table 1). The mean trophic AA imbalance (expressed as the difference in trophic AA mol% in diet vs. consumer) was greatest between fish muscle and the Vegi-Pro

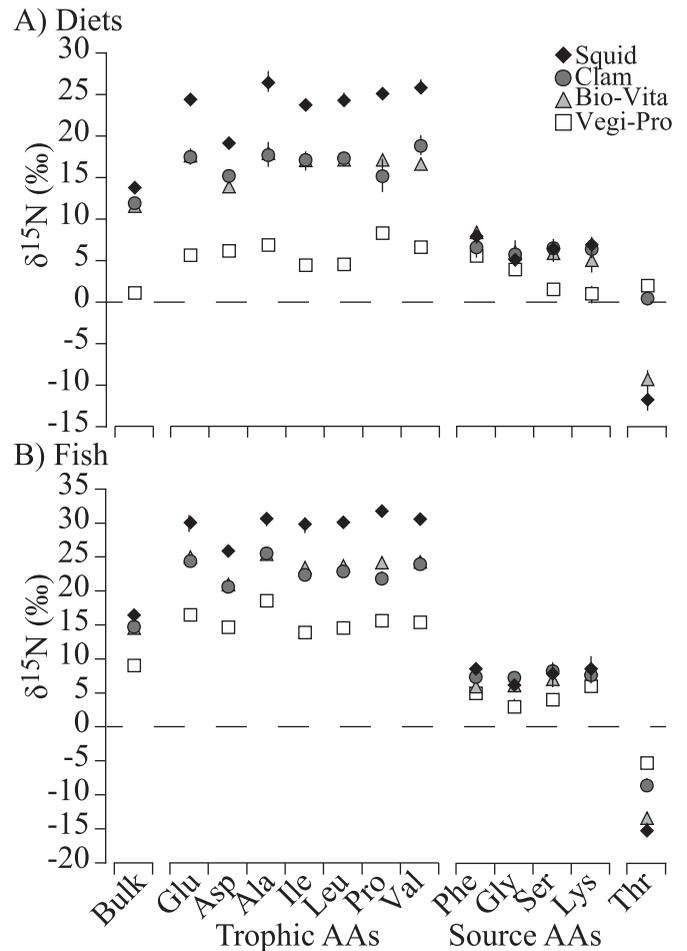


Fig. 1. Bulk tissue and individual AA $\delta^{15}\text{N}$ values (mean \pm SD) of (A) four diet types ($n = 3$ aliquots per diet type) and (B) *F. heteroclitus* muscle ($n = 3$ tank means per diet type) fed the four diet types: Vegi-Pro (open squares), Bio-Vita (light gray triangles), clam (dark gray circles), and squid (black diamonds). Abbreviations: Glu, glutamic acid; Asp, aspartic acid; Ala, alanine; Ile, isoleucine; Leu, leucine; Pro, proline; Val, valine; Gly, glycine; Ser, serine; Lys, lysine; Phe, phenylalanine; Thr, threonine.

($-1.3\% \pm 0.8\%$) and Bio-Vita ($-1.1\% \pm 0.2\%$) diets, where as the clam ($-0.1\% \pm 0.6\%$) and squid ($0.0\% \pm 0.6\%$) diets generally had trophic AA mol% values in balance with fish muscle.

The $\delta^{15}\text{N}$ values of individual trophic AAs were highly variable among diet treatments, while source AA values varied little in comparison (Fig. 1). The range in individual AA $\Delta^{15}\text{N}_{\text{C-D}}$ values (-9.0% for Thr in the clam diet to 11.7% for Ala in Vegi-Pro) was considerably larger than that of bulk trophic fractionation (2.6% for clam to 7.9% for Vegi-Pro) (Fig. 2). All individual AAs that were *a priori* identified as trophic AAs (Glu, Asp, Ala, Leu, Ile, Pro, Val) had $\Delta^{15}\text{N}_{\text{C-D}}$ values significantly different from 0‰ (Table 2). The $\Delta^{15}\text{N}_{\text{C-D}}$ values for the trophic AAs were generally both large and strikingly homogenous across individual AAs within a diet

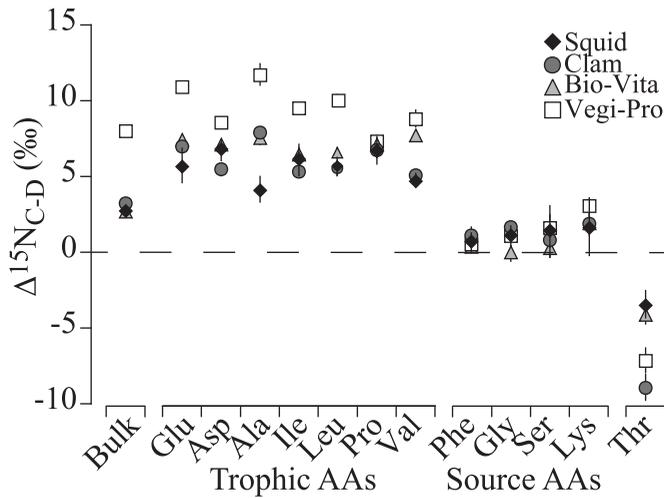


Fig. 2. Bulk tissue and individual AA stable nitrogen isotope trophic fractionation (mean $\Delta^{15}\text{N}_{\text{C-D}} \pm \text{SD}$) between diet and consumer for *F. heteroclitus* fed four diets differing in protein content and AA composition: Vegi-Pro (open squares), Bio-Vita (light gray triangles), clam (dark gray circles), and squid (black diamonds) ($n = 3$ tank means per diet type). See Fig. 1 for AA abbreviations.

Table 2. Results of one sample *t*-tests to determine if individual AA $\Delta^{15}\text{N}_{\text{C-D}}$ values averaged across diet types (mean \pm SD, $n =$ four diet types) were significantly different from 0‰.

	$\Delta^{15}\text{N}_{\text{C-D}}$	<i>t</i> (df = 3)
Glutamic acid†	7.7 \pm 2.2	6.9**
Aspartic acid†	6.9 \pm 1.3	11.0**
Alanine†	7.8 \pm 3.1	5.0*
Isoleucine†	6.8 \pm 1.8	7.4**
Leucine†	6.9 \pm 2.1	6.7**
Proline†	6.9 \pm 0.3	43.7***
Valine†	6.5 \pm 2.0	6.4**
Glycine#	0.9 \pm 0.7	2.5 ^{ns}
Serine#	0.9 \pm 0.6	3.1 ^{ns}
Lysine#	2.1 \pm 0.6	6.6**
Phenylalanine#	0.5 \pm 0.4	2.9 ^{ns}
Threonine	-6.0 \pm 2.6	-4.6*

Trophic AAs are indicated by † and source AAs are indicated by #. ^{ns} $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

type (mean $\Delta^{15}\text{N}_{\text{C-D}} = 7.1 \pm 0.5\text{‰}$). However, there were significant differences in trophic AA $\Delta^{15}\text{N}_{\text{C-D}}$ values among diet types, including the canonical trophic AA Glu (Table 3). Conversely, all individual AAs except Lys (mean $\Delta^{15}\text{N}_{\text{C-D}} = 2.1\text{‰} \pm 0.6\text{‰}$) that were *a priori* identified as source AAs (Gly, Ser, Phe) had $\Delta^{15}\text{N}_{\text{C-D}}$ values that were not significantly different from 0‰ (Table 2). Phenylalanine, in particular, had mean $\Delta^{15}\text{N}_{\text{C-D}}$ values ($\Delta^{15}\text{N}_{\text{C-D}} = 0.5\text{‰} \pm 0.4\text{‰}$) closest to 0‰ (Table 2), with no significant differences among dietary treatments (Table 3). Based on these data we used Phe as the

canonical source AA for all subsequent analyses of TDFs. Finally, Thr exhibited unique behavior outside of either category, showing negative $\Delta^{15}\text{N}_{\text{C-D}}$ values ($-6.0\text{‰} \pm 2.6\text{‰}$) between trophic levels.

Trophic discrimination factors ($\text{TDF}_{\text{AA-Phe}}$), calculated from individual trophic AA $\Delta^{15}\text{N}_{\text{C-D}}$ values normalized to Phe $\Delta^{15}\text{N}_{\text{C-D}}$ values in Eq. 4, showed large variations across diet types with values ranging from 3.5‰ for Ala in the squid treatment to 11.3‰ for Ala in the Vegi-Pro treatment (Table 4). We found a significant effect of diet on all $\text{TDF}_{\text{AA-Phe}}$ values with the exception of $\text{TDF}_{\text{Pro-Phe}}$ where the effect of diet was not significant (Table 4). Diet composition, expressed as a function of AA imbalance and protein content, was strongly correlated with $\text{TDF}_{\text{AA-Phe}}$ values for nearly all trophic AAs (Figs. 3, 4). This was particularly noticeable for the commonly used $\text{TDF}_{\text{Glu-Phe}}$ parameter, which showed significant negative linear relationships with both AA imbalance (Fig. 3a) and diet protein content (4a). Proline (Figs. 3f, 4f) was the only trophic AA with $\text{TDF}_{\text{AA-Phe}}$ values that did not show significant linear relationships with either measure of diet quality.

Discussion

The analysis of N stable isotopes in specific AAs provides a powerful method for examining food web architecture in ocean ecosystems (reviewed in McMahon et al. 2013b). However, it has recently become clear that we still lack an understanding of the processes controlling N isotope fractionation in AAs during transfer across trophic levels within marine food webs (e.g., Lorrain et al. 2009; Dale et al. 2011; Choy et al. 2012). Our study found that $\text{TDF}_{\text{AA-Phe}}$, the critical parameter in TP_{CSIA} calculations, is in fact closely linked to two metrics of diet quality, diet protein content and AA imbalance between diet and consumer. The results suggest, then, that diet composition may need to be taken into account when estimating consumer TP_{CSIA} .

Fractionation factors for source and trophic AAs

Trophic fractionation factors ($\Delta^{15}\text{N}_{\text{C-D}}$) determined for the source AAs confirmed expectations of significantly lower changes in $\delta^{15}\text{N}$ values between diet and consumer compared to bulk SIA. However, among source AAs, Phe was notable in our study for having $\Delta^{15}\text{N}_{\text{C-D}}$ values closest to 0‰ and with lowest variance across all diets. The mean $\Delta^{15}\text{N}_{\text{C-D}}$ value obtained for Phe in our study ($0.5\text{‰} \pm 0.4\text{‰}$) was almost identical to the value Chikaraishi et al. (2009) obtained from a very different group of marine food sources and consumers ($0.4\text{‰} \pm 0.5\text{‰}$). It has previously been hypothesized that the low $\Delta^{15}\text{N}_{\text{C-D}}$ value for Phe is related to details of its metabolic processing in animals, specifically the fact that the conversion of Phe to tyrosine via phenylalanine 4-monooxidase does not form or break any C–N bonds (Bender 2002; Chikaraishi et al. 2007). Our results support the validity of using $\delta^{15}\text{N}$ values of Phe to assess the foraging

Table 3. $\Delta^{15}\text{N}_{\text{C-D}}$ values (mean \pm SD, $n = 3$ tanks per treatment) for *F. heteroclitus* fed four diets differing in protein content and AA composition.

	Vegi-Pro	Bio-Vita	Clam	Squid	F_{df}
Glutamic acid†	10.8 \pm 0.2 ^a	7.3 \pm 0.3 ^b	6.9 \pm 0.3 ^{bc}	5.6 \pm 1.1 ^c	$F_{3,8} = 40.2^{***}$
Aspartic acid†	8.5 \pm 0.0 ^a	7.0 \pm 0.2 ^b	5.4 \pm 0.3 ^c	6.7 \pm 0.7 ^b	$F_{3,8} = 32.2^{***}$
Alanine†	11.7 \pm 0.7 ^a	7.5 \pm 0.4 ^b	7.8 \pm 0.2 ^b	4.1 \pm 0.8 ^c	$F_{3,8} = 85.9^{***}$
Isoleucine†	9.4 \pm 0.1 ^a	6.4 \pm 0.5 ^b	5.2 \pm 0.3 ^b	6.1 \pm 1.0 ^b	$F_{3,8} = 28.8^{***}$
Leucine†	10.0 \pm 0.3 ^a	6.6 \pm 0.3 ^b	5.5 \pm 0.5 ^b	5.7 \pm 0.6 ^b	$F_{3,8} = 65.8^{***}$
Proline†	7.3 \pm 0.4 ^a	7.0 \pm 0.6 ^a	6.7 \pm 0.8 ^a	6.6 \pm 0.4 ^a	$F_{3,8} = 0.9^{\text{ns}}$
Valine†	8.8 \pm 0.5 ^a	7.6 \pm 0.3 ^b	5.0 \pm 0.3 ^c	4.6 \pm 0.4 ^c	$F_{3,8} = 77.2^{***}$
Glycine [#]	1.0 \pm 0.2 ^{ab}	-0.1 \pm 0.5 ^a	1.6 \pm 0.2 ^b	1.0 \pm 0.7 ^{ab}	$F_{3,8} = 7.3^*$
Serine [#]	1.5 \pm 0.9 ^a	0.2 \pm 0.5 ^a	0.7 \pm 0.4 ^a	1.4 \pm 1.7 ^a	$F_{3,8} = 1.1^{\text{ns}}$
Lysine [#]	3.0 \pm 0.5 ^a	1.8 \pm 0.3 ^a	1.8 \pm 0.7 ^a	1.6 \pm 1.9 ^a	$F_{3,8} = 1.0^{\text{ns}}$
Phenylalanine [#]	0.4 \pm 0.4 ^a	0.1 \pm 0.3 ^a	1.0 \pm 0.6 ^a	0.6 \pm 0.6 ^a	$F_{3,8} = 1.6^{\text{ns}}$
Threonine	-7.2 \pm 0.8 ^a	-4.1 \pm 0.6 ^b	-9.0 \pm 0.9 ^a	-3.5 \pm 0.9 ^b	$F_{3,8} = 31.7^{***}$

Trophic AAs are indicated by † and source AAs are indicated by #. Different superscript letters indicate significant differences among treatments according to separate one-way ANOVAs with Tukey's HSD post hoc tests, ^{ns} $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Table 4. Trophic discrimination factors (mean $\text{TDF}_{\text{AA-Phe}} \pm$ SD, $n = 3$ tanks per treatment) for *F. heteroclitus* fed four diets differing in protein content and AA composition.

	Vegi-Pro	Bio-Vita	Clam	Squid	F_{df}
Glutamic acid†	10.4 \pm 0.6 ^a	7.2 \pm 0.6 ^b	5.9 \pm 0.4 ^{bc}	5.1 \pm 0.7 ^c	$F_{3,8} = 51.7^{***}$
Aspartic acid†	8.1 \pm 0.4 ^a	6.9 \pm 0.4 ^b	4.4 \pm 0.4 ^c	6.1 \pm 0.2 ^b	$F_{3,8} = 60.0^{***}$
Alanine†	11.3 \pm 0.8 ^a	7.3 \pm 0.2 ^b	6.8 \pm 0.4 ^b	3.5 \pm 0.8 ^c	$F_{3,8} = 81.0^{***}$
Isoleucine†	9.0 \pm 0.4 ^a	6.2 \pm 0.4 ^b	4.2 \pm 0.4 ^c	5.5 \pm 0.4 ^b	$F_{3,8} = 76.5^{***}$
Leucine†	9.6 \pm 0.6 ^a	6.4 \pm 0.6 ^b	4.5 \pm 0.2 ^c	5.1 \pm 0.4 ^c	$F_{3,8} = 66.5^{***}$
Proline†	6.9 \pm 0.6 ^a	6.9 \pm 0.8 ^a	5.7 \pm 0.8 ^a	6.0 \pm 0.9 ^a	$F_{3,8} = 1.9^{\text{ns}}$
Valine†	8.4 \pm 0.7 ^a	7.5 \pm 0.5 ^a	4.0 \pm 0.4 ^b	4.0 \pm 0.2 ^b	$F_{3,8} = 69.7^{***}$

$\text{TDF}_{\text{AA-Phe}}$ values were calculated for all trophic AAs normalized to phenylalanine. Different superscript letters indicate significant differences among treatments according to separate one-way ANOVAs with Tukey's HSD post hoc tests, ^{ns} $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

behavior of animals and shifts in $\delta^{15}\text{N}_{\text{baseline}}$ values across a wide range of species and ecosystems, including penguins (Lorrain et al. 2009, 2014), teleost fishes (Popp et al. 2007), elasmobranchs (Dale et al. 2011), deep-sea corals (Sherwood et al. 2011, 2014), and pinnipeds (Germain et al. 2013).

Among other source AAs, previous studies have shown that both methionine and tyrosine can have very low $\Delta^{15}\text{N}_{\text{C-D}}$ values, possibly owing to similar metabolic processing that does not involve transamination/deamination (McClelland and Montoya 2002; Chikaraishi et al. 2007). However, these AAs are not particularly amenable to routine CSIA analysis due to generally low abundance in animal tissues and poor chromatography with most common separation methods. Glycine and serine are also typically classified as source AAs, despite their potential to be transaminated/deaminated. These AAs, however, can have highly variable $\Delta^{15}\text{N}_{\text{C-D}}$ values, ranging from $<1\text{‰}$ to $>8\text{‰}$ across a range of different species (see Fig. 5 in Chikaraishi et al. 2009 and references

therein). The $\Delta^{15}\text{N}_{\text{C-D}}$ values for Gly and Ser obtained in our feeding experiments were also higher and more variable than that for Phe, further suggesting that these AAs should likely not be used as proxies for $\delta^{15}\text{N}_{\text{baseline}}$ values. Overall, our results support the conclusion that Phe is the best tracer for $\delta^{15}\text{N}$ values at the base of ocean food webs.

The observation that all trophic AAs had significantly larger $\Delta^{15}\text{N}_{\text{C-D}}$ values than bulk across all diet types agreed well with expectations from previous controlled feeding experiments in both marine (McClelland and Montoya 2002; Chikaraishi et al. 2009; Bloomfield et al. 2011; Germain et al. 2013; Miller et al. 2013; Bradley et al. 2014; Nakatomi et al. 2014) and terrestrial ecosystems (Chikaraishi et al. 2010, 2011; Nakashita et al. 2011; Steffan et al. 2013). Almost all experimental studies to date have found that Glu consistently had the largest $\Delta^{15}\text{N}_{\text{C-D}}$ values, and researchers concluded that this AA is the most sensitive proxy for trophic transfer. However, we found a significant effect of

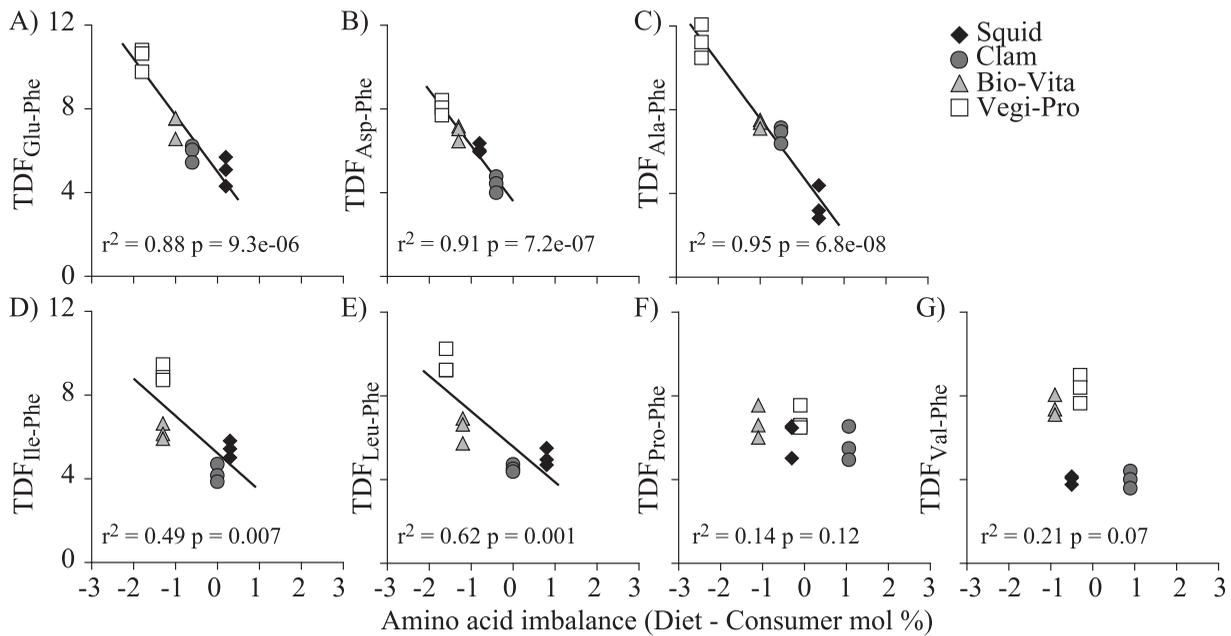


Fig. 3. The linear relationship between AA imbalance (individual AA mol percent abundance in diet minus consumer) and trophic discrimination factors (mean $TDF_{AA-Phe} \pm SD$) of (A) glutamic acid, (B) aspartic acid, (C) alanine, (D) isoleucine, (E) leucine, (F) proline, and (G) valine for *F. heteroclitus* fed four diets of different compositions (Vegi-Pro: squares, Bio-Vita: triangles, clam: circles, and squid: diamonds) ($n = 12$ tanks). Negative values for AA imbalance signify a lower mol percent abundance in the diet relative to the fish muscle. r^2 and p value are from linear regressions.

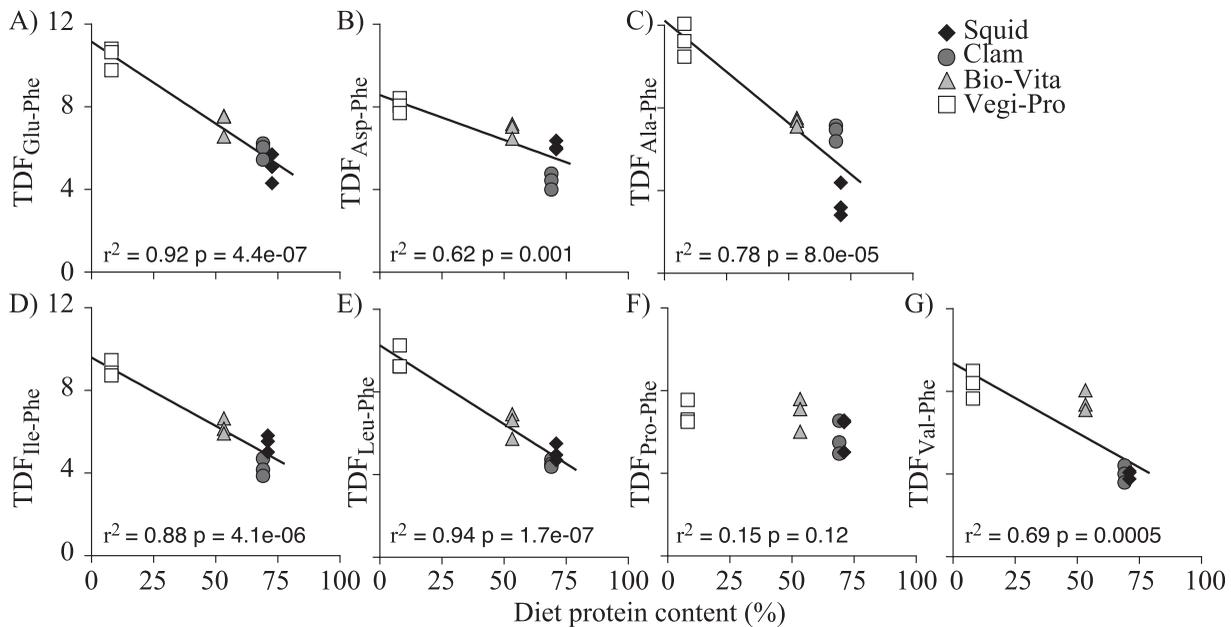


Fig. 4. The linear relationship between diet protein content (%) and trophic discrimination factors (mean $TDF_{AA-Phe} \pm SD$) of (A) glutamic acid, (B) aspartic acid, (C) alanine, (D) isoleucine, (E) leucine, (F) proline, and (G) valine for *F. heteroclitus* fed four diets of different compositions (Vegi-Pro: squares, Bio-Vita: triangles, clam: circles, and squid: diamonds) ($n = 12$ tanks). r^2 and p value are from linear regressions.

diet composition on $Glu \Delta^{15}N_{C-D}$, which represents a potential problem when using these values to determine trophic levels of consumers. In our study, values of $Glu \Delta^{15}N_{C-D}$ (mean $7.7\text{‰} \pm 2.2\text{‰}$) ranged from 5.7‰ to 10.8‰ across diet

treatments. This variability was, in turn, linked to measures of diet quality as discussed in the following section, which may provide a potential mechanism behind recent field observations that TP_{CSIA} calculations appear less accurate for

higher trophic level consumers (Lorrain et al. 2009; Dale et al. 2011; Choy et al. 2012).

Trophic discrimination factors and diet composition

In our controlled feeding experiment, we examined single trophic transfers using the same consumer species, but with diets varying in composition and quality. This study design allowed us to specifically examine the hypothesis that variability in diet quality (protein content and AA composition) represents an underlying mechanism influencing TDF_{AA-Phe} values, and therefore, the accuracy of TP estimates. We found significant relationships between TDF_{AA-Phe} values and two key metrics of diet quality (protein content and AA composition). In both cases, $TDF_{Glu-Phe}$ values trended progressively lower than the literature $TDF_{Glu-Phe}$ value of 7.6‰ with higher quality diets (i.e., both higher protein content and smaller AA imbalance between diet and consumer). The lower $TDF_{Glu-Phe}$ values with increasing diet quality are most likely due to decreased deamination of Glu and loss of ^{14}N during excretion for fishes feeding on high quality diets. These results align well with previous bulk SIA work, which has shown that animals consuming diets high in protein content (i.e., high quantity) with AA compositions that match that of their own body tissues (i.e., high quality) typically have lower bulk $\Delta^{15}N_{C-D}$ values due to decreased AA scavenging (Robbins et al. 2005, 2010; Mill et al. 2007; Florin et al. 2011). In addition, consumption and excretion rates are typically significantly lower for carnivorous fishes at higher trophic levels compared to herbivorous fishes (Clements et al. 2009), which has also been shown to significantly lower bulk trophic fractionation (Mill et al. 2007).

The very high $TDF_{Glu-Phe}$ values for fish fed the terrestrial plant based Vegi-Pro diet (10.4‰) likely represent an extreme end member for marine systems. Nonetheless, our observation further supports the general trends that $TDF_{Glu-Phe}$ varied significantly with diet quality. The biochemical composition of terrestrial and marine production is typically quite different (e.g., Clements et al. 2009), and the terrestrial plant diet used in this study had a very low percent protein (Vegi-Pro = 8% protein) coupled with large AA abundance deficits relative to consumer tissues. While there are few similar experiments with which to compare our results, Bloomfield et al. (2011) found a similarly high $TDF_{Glu-Phe}$ value (10‰) for the teleost fish black bream (*Acanthopagrus butcheri*) fed a vegetable-based diet.

Fish fed the Bio-Vita diet in our study had $TDF_{Glu-Phe}$ values (7.2‰) similar to the 7.6‰ value shown for lower trophic level consumers in a number of previous studies, both in marine (McClelland and Montya 2002; Chikaraishi et al. 2007, 2009) and terrestrial ecosystems (Chikaraishi et al. 2010, 2011; Steffan et al. 2013). Fish in our study that were fed clam and squid diets with high protein content and AA compositions similar to the fish had $TDF_{Glu-Phe}$ values (clam $TDF_{Glu-Phe} = 5.9‰$, squid $TDF_{Glu-Phe} = 5.1‰$) significantly

below 7.6‰. Similarly low $TDF_{Glu-Phe}$ values have been reported in a number of recent experiments on organisms feeding at higher trophic levels (Germain et al. 2013; Bradley et al. 2014; Hoen et al. 2014; McMahon et al. 2015). Our results suggest that these low $TDF_{Glu-Phe}$ values may be explained by diet composition and quality.

Interestingly, several previous studies on terrestrial food webs have found very consistent $TDF_{Glu-Phe}$ values near 7.6‰ across a number of trophic transfers (Chikaraishi et al. 2010, 2011; Steffan et al. 2013). However, these studies also found patterns of individual AA $\Delta^{15}N_{C-D}$ that were quite often different from marine studies (Chikaraishi et al. 2010, 2011; Steffan et al. 2013). For instance, while Steffan et al. (2013) found consistent $TDF_{Glu-Phe}$ values of 7.6‰ across three trophic transfers in a terrestrial insect food web, the study found trophic fractionation of source AAs significantly different from 0‰ (e.g., $\Delta^{15}N_{Phe}$ of $-4.5‰ \pm 0.8‰$). This pattern is quite different from most marine studies that found source AAs, particularly Phe, to have little to no trophic fractionation, and thus reflect $\delta^{15}N_{baseline}$. Clearly, more work is needed to understand the underlying mechanisms that lead to consistent trophic discrimination across trophic levels in terrestrial systems (Chikaraishi et al. 2010, 2011; Steffan et al. 2013) yet substantial changes in trophic discrimination across trophic levels in marine systems (Lorrain et al. 2009; Dale et al. 2011; Choy et al. 2012; Germain et al. 2013; Bradley et al. 2014; Hoen et al. 2014; McMahon et al. 2015; This study).

While our data strongly support the hypothesis of variable $TDF_{Glu-Phe}$ values linked directly to diet quality, there are several alternative hypotheses that might also contribute to lower $\Delta^{15}N_{Glu}$ values (and thus $TDF_{Glu-Phe}$ values) for specific higher trophic level consumers. Prior work with bulk SIA suggests that $\Delta^{15}N_{C-D}$ values are typically smaller in rapidly growing individuals (reviewed in Waters-Rist and Katzenberg 2010). While it is possible that trophic AAs could therefore also be less enriched in rapidly growing animals, this mechanism cannot explain variability in $TDF_{Glu-Phe}$ values across diet types for fish of the same age and growth rate in our study. Germain et al. (2013) also found similar and low $TDF_{Glu-Phe}$ values for both fast-growing juvenile seals and slower-growing adult seals.

Germain et al. (2013) found $TDF_{Glu-Phe}$ values (4.3‰) for ureotelic harbor seals fed high quality herring diets that were even lower than the $TDF_{Glu-Phe}$ values for ammonotelic fishes (5.1‰) in our study. They hypothesized that nitrogen excretion mode might be an important variable in determining $TDF_{Glu-Phe}$ values, specifically for marine mammals. In most previous literature at that time, low $TDF_{Glu-Phe}$ values had been observed only in higher trophic level consumers that were also urea/uric acid-producers [e.g., seals (Zhao 2002; Germain et al. 2013), penguins (Lorrain et al. 2009)], or which possessed urea-retention mechanisms [e.g., elasmobranchs (Dale et al. 2011)]. In contrast, lower trophic level

consumers (with higher $TDF_{Glu-Phe}$ values) were uniformly ammonotelic. This explanation remains plausible, relying on divergences in biochemical pathways, residence times for different reservoirs, and kinetic isotope effects between AA pools to explain the differences in $TDF_{Glu-Phe}$ between ammonia and urea excreting animals. However, it clearly cannot be the major driving mechanism for the low $TDF_{Glu-Phe}$ values in our study and that of Hoen et al. (2014) working on ammonotelic teleost fishes. For ureotelic animals, diet quality and nitrogen excretion mechanisms are not mutually exclusive. However, we suggest that the clear linkage to diet quality shown here may be able to explain much of the $TDF_{Glu-Phe}$ variability in higher TP consumers.

Most TDF_{AA-Phe} values in our study (mean $7.2\% \pm 2.4\%$), including the most commonly used $TDF_{Glu-Phe}$, were significantly influenced by one or both measures of diet quality in our study. However, $TDF_{Pro-Phe}$ showed considerably less variability with diet composition (mean $6.4\% \pm 0.6\%$). Chikaraishi et al. (2009) proposed that Pro might usefully replace Glu in TP calculations, although they noted that precision was generally better when Glu was used. We assembled $TDF_{Pro-Phe}$ values from a number of previously published controlled feeding experiments across a range of taxa and found general agreement with the results from our experiment. Reported values of $TDF_{Pro-Phe}$ ranged from 4.9% in Gentoo penguins fed fish (McMahon et al. 2015), $5.6\text{--}5.8\%$ for zooplankton feeding on phytoplankton (McClelland and Montoya 2002; Chikaraishi et al. 2009), 6.1% for a gastropod grazing on macroalgae (Chikaraishi et al. 2007) and finally 7.0% in seals fed fish (Germain et al. 2013). The fact that $TDF_{Pro-Phe}$ values are relatively similar among a number of different consumer taxa, trophic levels, and diet types, combined with our data showing little variation in $TDF_{Pro-Phe}$ as a function of diet quality, suggest that further investigation into the use of Pro as an alternative to Glu in TP calculations is warranted.

Our study provides evidence for shifts in diet composition and quality as a major underlying mechanism for changes in $TDF_{Glu-Phe}$ values across TPs in aquatic food webs. As such, our results suggest that a single $TDF_{Glu-Phe}$ value is unlikely to be appropriate for reconstructing TP_{CSIA} in systems where the average diet composition and quality varies across trophic levels. We propose that this effect may underlie recent findings of underestimation of TP_{CSIA} at higher trophic levels for studies using the conventional $TDF_{Glu-Phe}$ value of 7.6% (Lorrain et al. 2009; Dale et al. 2011; Choy et al. 2012). For example, Choy et al. (2012) used CSIA ($TDF_{Glu-Phe} = 7.6\%$) to calculate the TP of two groups of mesopelagic fishes. Lanternfishes (family Myctophidae) are zooplanktivorous secondary consumers (expected TP = 3.2 from 361 published food item records), and dragonfishes (family Stomiidae) are piscivorous tertiary consumers (expected TP = 4.0 from 73 published food item records). The authors found that estimated TP_{CSIA} calculated with a single $TDF_{Glu-Phe}$

value of 7.6% for lanternfishes largely aligned with expectations from stomach content studies, as would be predicted from our study results. However, TP_{CSIA} for dragonfishes were nearly a full trophic level lower than expected when using the conventional $TDF_{Glu-Phe}$ of 7.6% . Our results suggest that this discrepancy may be because dragonfishes, which feed primarily on high quality fish diets, have lower $TDF_{Glu-Phe}$ values than lanternfishes that feed primarily on zooplankton with a greater AA imbalance between diet and consumer. If we recalculate the TP_{CSIA} of dragonfishes in the Choy et al. (2012) study using a multi- $TDF_{Glu-Phe}$ that includes the high quality diet $TDF_{Glu-Phe}$ of 5.1% from our study, we get a mean TP_{CSIA} for dragonfishes of 3.8 that is nearly identical to the expected TP of 4.0 for this piscivorous consumer. This example illustrates the potential advantage of taking diet composition into account when calculating the TP_{CSIA} of marine consumers. We also suggest further investigation into the use of Pro as a trophic AA in TP_{CSIA} calculations as a way to limit the effects of variable diet composition on trophic fractionation. Together, the findings from this study point to potentially significant refinements to the application of CSIA in studies of resource acquisition and allocation, trophic dynamic, and food web architecture in aquatic ecosystems.

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