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Trophic position estimates of marine teleosts using amino acid compound specific isotopic analysis

Christina J. Bradley,[†*1] Natalie J. Wallsgrove,[2] C. Anela Choy,[1] Jeffrey C. Drazen,[1] Elizabeth D. Hetherington,[3,4] Danielle K. Hoen,[1] Brian N. Popp[2]

[1]Department of Oceanography, University of Hawaii, Honolulu, Hawaii
[2]Department of Geology and Geophysics, University of Hawaii, Honolulu, Hawaii
[3]Division of Biological Sciences, University of California, San Diego, La Jolla, California
[4]Department of Marine Science and Environmental Studies, University of San Diego, San Diego, California

Abstract

The increasing use of trophic position (TP) for assessing and describing ecosystems has resulted in the emergence of novel techniques for accurate and accessible measurements. The strength of amino acid compound specific nitrogen isotopic analysis (AA-CSIA) lies in its ability to determine TP using a single organism, utilizing the isotopic differences between two or more amino acids (AAs). However, calculating TP from this technique relies upon the predictability of isotopic differences across consumer types and consistency among ecosystems. The goal of this study was to use wild samples to evaluate ^{15}N enrichment between AA groups with differing trophic fractionation patterns, termed the trophic discrimination factor (TDF_{AA}), and determine an accurate value for calculating TPs of marine teleosts. Using a large sample size (224 samples of 47 species) across a broad range of TPs (2.0–4.5, estimated by stomach content analysis [SCA]) and marine ecosystems, we derived TDF_{AA} values that explained up to 80% of the variability between TP calculations from SCA and AA-CSIA. We found a TDF_{AA} value of $5.7 \pm 0.3‰$ using the difference between $\delta^{15}N$ values of glutamic acid and phenylalanine, 2‰ lower than the most commonly used value of $7.6 \pm 1.3‰$. The weighted mean of several AAs resulted in the same value for TDF_{AA}, but provided greater agreement with TPs from SCA. For more reliable TP calculations in marine teleosts, our results strongly advocate a lower TDF_{AA} and the use of weighted mean AA $\delta^{15}N$ values from triplicate analyses.

Traditional methods for calculating trophic position (TP) include stomach content analysis (SCA) and stable isotope analysis (SIA). SCA has been valued for taxonomic resolution of diets, identifying prey items and their relative importance, and using this information to calculate TP (Hyslop 1980). However, SCA may be biased by what dietary items are identifiable in the food bolus and can be misleading from an energetics perspective because not all dietary material is actually assimilated (Rindorf and Lewy 2004). Furthermore, SCA only presents a snapshot of the most recent foraging events which can be problematic for opportunistic marine consumers. Despite these known limitations, SCA has been the conventional method for establishing stomach content derived trophic position (TP_{SCA}) of fish for decades (e.g., Hynes 1950) because it provides a level of taxonomic resolution that cannot be achieved using other methods. Time-integrated information about long-term nutrient assimilation can be measured using $\delta^{15}N$ values of whole organisms or their bulk tissues (Olson et al. 2010). These values are then used to estimate TP based on the observation that $\delta^{15}N$ values predictably increase (2–4‰) with each trophic level (Pinnegar and Polunin 1999; Vander Zanden and Rasmussen 2001; Post 2002; Buchheister and Latour 2010). However, different nitrogen sources for primary producers (e.g., NH_4^+, NO_3^-, N_2) can have different $\delta^{15}N$ values that influence the nitrogen isotopic composition of consumers yielding variable $\delta^{15}N$ values spatially and temporally within an ecosystem that are unrelated to TP (Post 2002). Constraining the nitrogen isotopic baseline, or isotopic composition of primary producers at the base of an ecosystem, can be complicated and may be difficult or impossible in many environments (Popp et al. 2007). Although SCA and SIA have inherent limitations, when used in combination these methods have been shown to be highly complementary and can enhance

*Correspondence: cbradley2@ucmerced.edu

[†]Present address: School of Natural Sciences, University of California, Merced, California, 95343

the accuracy of TP estimates (de la Moriniere et al. 2003; Ho et al. 2007; Pepin and Dower 2007).

Further attention has been given to determining TP in ecosystem studies using amino acid compound specific nitrogen isotopic analysis (AA-CSIA), namely for its ability to circumvent many of the limitations of SCA and bulk tissue SIA. More specifically, nitrogen isotopic composition distinguishes two classes of amino acids (AAs) which yield several pieces of information that can be applied to ecosystem studies. One group of AAs is designated as "source"; their isotopic compositions change little with increasing trophic steps and reflect baseline $\delta^{15}N$ values. The other group, the "trophic" AAs (*sensu* Popp et al. 2007) show large ^{15}N enrichment relative to "source" AAs (up to 8‰) with each trophic level increase (Chikaraishi et al. 2009). Because both isotopic baseline and fractionation information are retained in the nitrogen isotopic composition of AAs, results of AA-CSIA from a single consumer provide both an integrated measurement of TP and the $\delta^{15}N$ value at the base of the food web (McClelland and Montoya 2002).

Calculated TP from the differences between the $\delta^{15}N$ values of trophic and source AAs, TP_{CSIA}, has been used in a number of studies (McClelland and Montoya 2002; Chikaraishi et al. 2007, 2009, 2010; McCarthy et al. 2007; Popp et al. 2007). The most commonly used equation for doing so is:

$$TP_{CSIA} = [(\delta^{15}N_{Trp} - \delta^{15}N_{Src} - \beta)/TDF_{AA}] + 1, \quad (1)$$

where $\delta^{15}N_{Trp}$ and $\delta^{15}N_{Src}$ are the nitrogen isotopic compositions of selected trophic and source AAs, respectively (McClelland and Montoya 2002; Chikaraishi et al. 2009). The symbol β is the difference between the $\delta^{15}N$ values of trophic and source AAs in primary producers (trophic level = 1). The trophic discrimination factor (TDF_{AA}) is the average ^{15}N enrichment in one or more trophic AAs relative to source AAs per trophic level.

When determining TP_{CSIA}, β, and TDF_{AA} are defined as constant values both among species and across TPs. A value of β equal to 3.4 ± 0.9‰ based on AA-CSIA of 17 aquatic photoautotrophs from microalgae to macroalgae, has been measured using the difference between the $\delta^{15}N$ values of glutamic acid and phenylalanine (Chikaraishi et al. 2009, 2010). However, more recent isotopic analysis of glutamic acid (Glu) and phenylalanine (Phe) in cultured prokaryotes and eukaryotes suggests that β values may be more variable (mean $\beta = 1.2 \pm 3.2$‰, McCarthy et al. 2013). Additionally, values for β in C3 and C4 plants (-8.4 ± 1.6‰ and -0.4 ± 1.7‰, respectively) are significantly different from marine algae (Chikaraishi et al. 2010); any terrestrial inputs into marine diets, also including inputs from seagrasses (Vander Zanden et al. 2013), could result in variability in β. Chikaraishi et al. (2009) suggested using Phe as the representative source AA because of its abundance in consumer tissues and near zero isotopic change during trophic transfers (0.4 ± 0.5‰). In general, they found that the patterns of ^{15}N enrichment in each trophic AA relative to Phe were similar across the taxa analyzed. They also identified Glu as a good trophic AA because it exhibited the largest isotopic fractionation with each trophic step and lowest isotopic variation among organisms (8.0 ± 1.2‰).

Uncertainty in calculated TP_{CSIA} can be driven by variation in TDF_{AA} values, which have been examined in only a limited number of organisms, tissue types, and physiological conditions. A TDF_{AA} value (7.6 ± 1.3‰) was originally established from $\delta^{15}N$ values of Glu and Phe in controlled feeding experiments, where either zooplankton consumed green algae or newly hatched fish consumed zooplankton (McClelland and Montoya 2002; Chikaraishi et al. 2009). However, some recent publications have shown consistent underestimations of TP_{CSIA} across diverse animals groups, often at or near the top of the food web, pointing to inaccurate TDFs as a potential cause (Lorrain et al. 2009; Dale et al. 2011; Choy et al. 2012; Germain et al. 2013). Furthermore, variations in nitrogen isotope discrimination factors of bulk tissue have been shown between taxa, diets, and tissues, leading to the use of taxa-specific trophic discrimination factors (TDFs) when available (Mill et al. 2007; Wyatt et al. 2010; Dale et al. 2011; Kim et al. 2011; Codron et al. 2012). TDFs for bulk tissues average around 3–4‰ (Post 2002), but variability around this range has led to a desire for more accurate assessment of values.

The purpose of this study was to establish an accurate TDF_{AA} value for use in trophic ecosystem studies by utilizing wild samples to encompass natural ecological variation. To address that objective, we compared TP_{CSIA} to that from SCA in fishes across a range of feeding strategies and habitats. We measured the isotopic compositions of various trophic and source AAs of 47 teleost species from 22 families for which TP is reasonably well known based on SCA. By comparing ^{15}N trophic enrichments of combinations of AAs, we derived TDF_{AA} values that yield TP estimates in fishes that more closely resemble those determined from SCA, supporting the application of the AA-CSIA approach to marine trophic studies.

Materials and procedures

Sample collection

We collected over 200 fish from 47 species spanning 22 families, including herbivores, planktivores, invertebrate predators, and piscivores (Table 1). The majority of fish were taken over a 4-year period (2008–2012) from the main Hawaiian Islands in the North Pacific Subtropical Gyre (NPSG), although several were collected from other ocean basins. Fish were separated into four major groups based on the general ecosystem type in which they resided. Reef fish are all reef-associated and were collected from depths of

Table 1. Fish species and collection locations. Fish species collected, number sampled, and general location of collection. Locations include the Eastern Tropical North Pacific (ETNP), California Current (CC), Gulf of Mexico (GOM), Tasman Sea (TS), the Northern Mid-Atlantic Ridge (NMAR), and Hawaiian Islands, grouped by those in the main islands (MHI), the Northwest Hawaiian Islands (NWHI), and general locations within the North Pacific Subtropical Gyre (NPSG). Habitat groups chosen given similarity of residence and collection location. Feeding guilds are labeled as H (herbivores), O (ominivores), PK (planktivores), I (benthic invertivores), PI (piscivores), and C (cleaners), according to their dominant prey items. The number of stomachs sampled for SCA (nSCA) is given, listed "OBS" for feeding observations, or marked "NA" if no available studies were found.

Species	Habitat group	Feeding guild	Order	*n*	Location	TP_{SCA} (SD)	nSCA	Reference
Acanthurus nigrofuscus	Reef	H	Perciformes	3	Hawaii (MHI)	2.00 (0.00)	20; OBS.	Robertson and Gaines (1986), Fishelson et al. (1987), Montgomery et al. (1989), Purcell and Bellwood (1993)
Ctenochaetus strigosus	Reef	H	Perciformes	11	Hawaii (MHI)	2.00 (0.00)	OBS.; 15	Hobson (1974), Robertson and Gaines (1986)
Scarus dubius	Reef	H	Perciformes	1	Hawaii (MHI)	2.00 (0.00)	NA	FishBase
Scarus psittacus	Reef	H	Perciformes	3	Hawaii (MHI)	2.00 (0.00)	33	Bellwood and Choat (1990), Choat et al. (2004)
Centropyge potteri	Reef	O	Perciformes	16	Hawaii (MHI)	2.58 (0.26)	5	Hobson (1974)
Acanthurus thompsoni	Reef	PK	Perciformes	1	Hawaii (MHI)	3.40 (0.40)	8	Gerber and Marshall (1974), Hobson (1974)
Chaetodon miliaris	Reef	PK	Perciformes	6	Hawaii (MHI)	3.02 (0.12)	83	Hobson (1974), Ralston (1981)
Chromis verater	Reef	PK	Perciformes	6	Hawaii (MHI)	3.28 (0.36)	807	Swerdloff (1970), Hobson (1974), Randall (1985)
Dascyllus albisella	Reef	PK	Perciformes	6	Hawaii (MHI)	3.11 (0.36)	49	Hobson (1974), Mann and Sancho (2007)
Myripristis berndti	Reef	PK	Bercyiformes	5	Hawaii (MHI)	3.72 (0.57)	24	Hiatt and Strasburg (1960), Hobson (1974), Randall (1985)
Myripristis chryseres	Reef	PK	Bercyiformes	5	Hawaii (MHI)	4.04 (0.67)	NA	FishBase
Forcipiger flavissimus	Reef	I	Perciformes	4	Hawaii (MHI)	3.08 (0.28)	24	Hobson (1974), Harmelin-Vivien and Bouchon-Navaro (1983)
Forcipiger longirostris	Reef	I	Perciformes	5	Hawaii (MHI)	3.50 (0.50)	24	Hobson (1974), Harmelin-Vivien and Bouchon-Navaro (1983), Randall (1985)
Heteropriacanthus cruentatas	Reef	I	Perciformes	2	Hawaii (MHI)	3.75 (0.47)	21	Randall (1967), Hobson (1974)
Ostorhinchus maculiferus	Reef	I	Perciformes	1	Hawaii (MHI)	3.50 (0.51)	132	Chave (1978), Randall (1998)
Parupeneus multifasciatus	Reef	I	Perciformes	8	Hawaii (MHI)	3.46 (0.56)	38	Hobson (1974), Sorden (1982), Randall (1985)
Parupeneus porphyreus	Reef	I	Perciformes	1	Hawaii (MHI)	3.50 (0.62)	211	Mahi (1969), Hobson (1974), Sorden (1982)
Pristiapogon kallopterus	Reef	I	Perciformes	1	Hawaii (MHI)	3.50 (0.57)	162	Chave (1978), Randall (1998)
Pseudocheilinus evanidus	Reef	I	Perciformes	6	Hawaii (MHI)	3.50 (0.37)	NA	FishBase

TABLE 1. Continued

Species	Habitat group	Feeding guild	Order	*n*	Location	TP_{SCA} (SD)	nSCA	Reference
Sargocentron diadema	Reef	I	Bercyiformes	2	Hawaii (MHI)	3.41 (0.49)	40	Hiatt and Strasburg (1960), Hobson (1974)
Sargocentron ensifer	Reef	I	Bercyiformes	1	Hawaii (MHI)	4.02 (0.67)	NA	FishBase
Sargocentron xantherythrum	Reef	I	Bercyiformes	7	Hawaii (MHI)	3.46 (0.54)	17	Hobson (1974)
Sufflamen bursa	Reef	I	Tetradontiformes	1	Hawaii (MHI)	3.10 (0.38)	9	Hobson (1974)
Cephalopholis argus	Reef	PI	Perciformes	18	Hawaii (MHI)	4.48 (0.79)	241	Hobson (1974), Shpigel and Fishelson (1989), Dierking et al. (2009)
Labroides phthirophagus	Reef	C	Perciformes	2	Hawaii (MHI)	4.03 (0.67)	OBS	Hobson (1974), FishBase
Aprion virescens	Bottomfish	PI	Perciformes	2	Hawaii (NWHI)	3.95 (0.66)	1171	Haight et al. (1993), Kulbicki et al. (2005)
Epinephelus quernus	Bottomfish	PI	Perciformes	2	Hawaii (NWHI)	4.03 (0.67)	NA	FishBase
Etelis carbunculus	Bottomfish	PI	Perciformes	2	Hawaii (NWHI)	4.49 (0.79)	92	Haight et al. (1993)
Etelis coruscans	Bottomfish	PI	Perciformes	2	Hawaii (MHI)	4.46 (0.75)	40	Haight et al. (1993)
Pristipomoides filamentosus	Bottomfish	PI	Perciformes	11	Hawaii (MHI)	3.64 (0.49)	232	Haight et al. (1993)
Benthosema glaciale	Mesopelagic	PK	Myctophiformes	5	NMAR	3.00 (0.29)	762	Gjøsaeter (1973), Roe and Badcock (1984), Sameoto (1988)
Benthosema suborbitale	Mesopelagic	PK	Myctophiformes	4	GOM	3.40 (0.45)	472	Hopkins and Gartner (1992), Hopkins et al. (1996), McClain-Counts (2011)
Bolinichthys longipes	Mesopelagic	PK	Myctophiformes	4	Hawaii (NPSG)	3.10 (0.22)	153	Clarke (1980)
Lampanyctus australis	Mesopelagic	PK	Myctophiformes	3	TS	3.30 (0.40)	810	Williams et al. (2001) (810)
Myctophum nitidulum	Mesopelagic	PK	Myctophiformes	6	ETNP	3.40 (0.45)	299	Van Noord et al. (2013) (299)
Stenobrachius leucopsarus	Mesopelagic	PK	Myctophiformes	4	CC	3.20 (0.30)	629	Collard (1970), Pearcy et al. (1979), Beamish (1999), Suntsov and Brodeur (2008)
Symbolophorus reversus	Mesopelagic	PK	Myctophiformes	6	ETNP	3.20 (0.40)	234	Collard (1970), Van Noord (2013)
Chauliodus sloani	Mesopelagic	PI	Stomiiformes	7	TS, GOM, Hawaii (NPSG)	4.20 (0.70)	753	Clarke (1982), Hopkins et al. (1996), Sutton and Hopkins (1996), Butler et al. (2001), Williams et al. (2001)
Idiacanthus antrostomus	Mesopelagic	PI	Stomiiformes	2	CC	3.80 (0.60)	3	Borodulina (1972)
Idiacanthus fasciola	Mesopelagic	PI	Stomiiformes	1	Hawaii (NPSG)	3.90 (0.67)	309	Clarke (1982), Sutton and Hopkins (1996)
Stomias boa	Mesopelagic	PI	Stomiiformes	3	NMAR	4.00 (0.64)	5	Borodulina (1972), Mauchline and Gordon (1983)
Coryphaena hippurus	Pelagic	PI	Perciformes	3	Hawaii (NPSG)	4.40 (0.80)	846	Massuti et al. (1998), Oxenford and Hunte (1999), Moteki et al. (2001), Young et al. (2010)
Katsuwonus pelamis	Pelagic	PI	Perciformes	9	ETNP, Hawaii (NPSG)	3.80 (0.60)	2929	Alverson (1963), Bernard et al. (1985), Tanabe (2001)

TABLE 1. Continued

Species	Habitat group	Feeding guild	Order	*n*	Location	TP_{SCA} (SD)	nSCA	Reference
Lampris guttatus	Pelagic	PI	Lampridiformes	3	Hawaii (NPSG)	4.20 (0.62)	134	Choy et al. (2013)
Thunnus albaceres	Pelagic	PI	Perciformes	9	ETNP, Hawaii (NPSG)	4.30 (0.70)	8717	King and Ikehara (1956), Alverson (1963), Bernard et al. (1985), Olson and Boggs (1986), Kim et al. (1997), Sabatié et al. (2003), Young et al. (2010)
Thunnus obesus	Pelagic	PI	Perciformes	9	ETNP, Hawaii (NPSG)	4.50 (0.80)	510	King and Ikehara (1956), Kim et al. (1997), Sabatié et al. (2003), Young et al. (2010)
Xiphias gladius	Pelagic	PI	Perciformes	3	Hawaii (NPSG)	4.50 (0.60)	699	Hernandez-Garcia (1995), Sabatié et al. (2003), Markaida and Hochberg (2005), Young et al. (2010)

130 m or shallower using spear guns, mono-filament nets, and suction guns, by closed-circuit rebreather divers or manned submersibles. The bottomfish grouping contains specimens occurring from 100 m to 300 m. Species of lanternfish and dragonfish were grouped as mesopelagics, as they were collected using midwater trawling equipment (*see* Choy et al. 2012 for detailed collection methods), or dipnets in the open ocean. Finally, large, open-ocean predator species were included under the grouping pelagics. These fish were collected by trained fishery observers working onboard fishing vessels or from a local seafood wholesaler on Oahu, Hawaii, but all fish were captured in the central NPSG.

In larger fish, white muscle tissue was taken from a section under the first or second dorsal fin of the fish, after removing skin and scales. To obtain sufficient sample in smaller fish, filets of white muscle and skin were taken from the entire length of the fish. Tissue samples were frozen at −20°C until they were dried and ground into a fine powder prior to isotope analysis. Whole fish samples of mesopelagic fish were frozen in liquid nitrogen and transferred to −80°C for storage until use in isotopic analysis.

AA isotope analysis

Muscle tissue samples were hydrolyzed and AAs were derivatized prior to nitrogen isotope analysis using established methods (Popp et al. 2007; Hannides et al. 2009). Dried, ground tissue (~1–5 mg) was hydrolyzed (6 N HCl) at 150°C for 70 min (Cowie and Hedges 1992). Acid hydrolysis under these conditions converts glutamine and asparagine to glutamic and aspartic acid, and destroys tryptophan and cystine. Hydrolysates were dissolved in 0.01 N HCl, and then passed through a 0.45 μm hydrophilic filter, followed by cation-exchange chromatography (Dowex 50WX8-400) following Metges et al. (1996). Samples were then esterified by heating at 110°C for 60 min in 4: 1 isopropanol: acetyl chloride, after which AAs were acylated by heating at 100°C for 15 min in 3: 1 methylene chloride: trifluoracetic anhydride. Using a 2: 1 P-buffer: chloroform mix (1 M P-buffer: $KH_2PO_4 + Na_2HPO_4$ in Milli-Q water, pH 7), AAs were further purified by partitioning into chloroform and discarding any compounds dissolved in the aqueous phase (Ueda et al. 1989). A final acylation step was repeated and samples were stored in 3: 1 methylene chloride: TFAA at −20°C for up to 6 months. Samples were dried under N_2 at ambient temperature and dissolved in ethyl acetate just prior to isotope analysis.

The $\delta^{15}N$ values of derivatized samples were determined using a Delta V Plus mass spectrometer interfaced to a Trace GC gas chromatograph through a GC-C III combustion furnace (980°C), reduction furnace (650°C), and liquid nitrogen cold trap via a GC-C III interface. Samples were injected (split/splitless injector in splitless mode) onto a *forte* BPx5 capillary column (60 m × 0.32 mm × 1.0 μm film thickness) at an injector temperature of 180°C with a constant helium

flow rate of 1.4 mL min^{-1}. The column was initially held at 50°C for 2 min and then increased to 190°C at a rate of 8°C min^{-1}. Once at 190°C, the temperature was increased at a rate of 10°C min^{-1} to 300 C where it was held for 7.5 min. All samples were analyzed at least in triplicate and measured $\delta^{15}N$ values were normalized to the known nitrogen isotopic composition of internal reference compounds norleucine and aminoadipic acid coinjected with each sample. The standard deviation of $\delta^{15}N$ values derived from multiple analyses averaged 0.52‰ and ranged from 0.03‰ to 1.2‰.

The nitrogen isotopic composition of 16 AAs can be quantified using the methods described. Of those 16, our ability to quantify the $\delta^{15}N$ values of isoleucine, methionine, arginine, tyrosine, and histidine was inconsistent among samples analyzed and these AAs were therefore not considered further. The remaining 11 consisted of threonine (Thr), trophic AAs alanine (Ala), aspartic acid (Asp), glutamic acid (Glu), leucine (Leu), proline (Pro), and valine (Val), and source AAs glycine (Gly), lysine (Lys), phenylalanine (Phe), and serine (Ser). Weighted mean values for groups of trophic and source AAs were calculated

$$\delta^{15}N_{\bar{x}_w} = \frac{\sum \frac{\delta^{15}N_x}{\sigma_x^2}}{\sum \frac{1}{\sigma_x^2}}, \quad (2)$$

where $\delta^{15}N_x$ is the nitrogen isotopic composition of a specified AA within the grouping and σ_x is the standard deviation of the specific AA (Hayes et al. 1990). Errors were propagated using the measured reproducibility of sample injections for each AA and the variance of samples within a species.

TP estimations

TP_{SCA} was obtained for each fish species using appropriate literature citations when available. For some species, insufficient published diet information was available and TPs were obtained from the online database FishBase (http://www.fishbase.org). FishBase estimates TP using two methods. The first utilizes the mean TP of food items from diet studies weighted by the contribution of each item within the diet, adding 1 to this mean value for the TP of the fish. The second method uses a randomized sampling routine of individual food items, assigning arbitrary ranks to items and calculating the percent contribution through an empirical model. FishBase recommends the use of the former when diet compositions are known, as the latter incorporates multiple possibilities for diet composition creating significant error around the estimate. If the diet composition for a particular species was unknown, FishBase estimated TP from congeners and a relationship between size and TP. Published diet studies, where available, for all TP estimates are listed in Table 1.

Preliminary TP_{CSIA} were calculated using Eq. 1. Uncertainty in TP was calculated by propagation of errors (Dale et al. 2011; Choy et al. 2012; Blum et al. 2013) and is treated quantitatively using the analytical solution of differentiation of Eq. 1:

$$\sigma_{TP}^2 = \left(\frac{\partial TP}{\partial \delta^{15}N_{Trp}}\right)^2 \sigma^2_{\delta^{15}N_{Trp}} + \left(\frac{\partial TP}{\partial \delta^{15}N_{Src}}\right)^2 \sigma^2_{\delta^{15}N_{Src}} + \left(\frac{\partial TP}{\partial \beta}\right)^2 \sigma_\beta^2 + \left(\frac{\partial TP}{\partial TEF}\right)^2 \sigma^2_{TEF}, \quad (3)$$

where σ is standard deviation of trophic position, TP, $\delta^{15}N$ values for trophic, Trp, or source, Src, AAs, β, or TDF. Values for standard deviation of isotopic compositions of AAs were calculated from both analytical error and between specimens. Standard deviations for β and TDF_{AA} were obtained from published values (McClelland and Montoya 2002; Chikaraishi et al. 2009; McCarthy et al. 2013). A detailed description of the derivation can be found in an electronic appendix. Residuals from TP_{CSIA}, as compared to TP_{SCA}, were used to evaluate factors affecting the calculation through analyses of variance (Kruskal–Wallis) and post hoc comparisons (Wilcoxon Rank-Sum).

Statistical analyses

Statistical analyses were conducted primarily using MATLAB (MathWorks), except for regression analyses which were performed using a Deming regression from SigmaPlot (version 12.5, Systat Software, Inc.) to account for error associated with both dependent and independent variables. As TP_{SCA} is represented for each species by a single numerical value with error resulting from within species variation, we chose to combine isotopic values for each species into a single mean value with error propagated from analytical methods as well as individual samples.

Assessment

The TP of a marine organism describes the position in a food web at which an organism feeds. The most basic evaluation of the TP of a marine organism is therefore based on what that organism eats, effectively the product of the TPs of an organism's various prey items and the proportion of each item in the diet (e.g., Christensen and Pauly 1992). Consequently, study of diet and food habits of marine organisms through analysis of the contents of their stomachs has been standard practice for many years (Hyslop 1980; Hynes 1950) and specific methods exist on how to obtain, analyze and report stomach content data (e.g., Cortes 1997). This kind of information allows recognition of trophic links within an ecosystem and identifies the pathways of energy and mass transfer within the community.

Quantification of the TP_{SCA} of a marine organism is not always straightforward and several critical reviews of this subject exist (Hyslop 1980; Cortes 1997; Hynes 1950; Baker et al. 2014). Techniques rely on the positive identification of

some body part of the prey and the contribution of each prey item to diet by weight (or volume) and number is used to establish the composition of diet. Nonetheless, significant uncertainties can exist and examination of a large number of individuals is required making SCA a labor-intensive and taxonomically specialized method (Hyslop 1980). To determine the TP of the consumer, diet composition and the mean TP of prey items must be known, a source of error that is compounded with increasing TP. SCA reveals only the most recent meal eaten and may be misleading because not all dietary material is actually assimilated. It may also be biased by what dietary items are identifiable in the food bolus. For instance, certain prey groups containing hard parts are often the only identifiable remains but soft bodied prey, which digest faster, could be an equal or even greater proportion of the consumer's diet. In addition to relative diet composition, varying (potentially species-specific) rates and levels of digestion affect the accuracy and taxonomic level with which any prey can be recognized and their TP identified (Hyslop 1980; MacDonald et al. 1982). Although SCA methods may be imperfect and many estimates approach an uncertainty of ±0.5 TP, in this article, we follow the approach taken by Fry (1988) and compare the fractional TP_{CSIA} to TP_{SCA}.

Derivation of β and TDF_{AA} from regression analysis

Traditionally, the determination of TDF_{AA} and β values involves calculation from nitrogen isotopic compositions of prey and consumer tissues in controlled feeding experiments, which in some cases may not replicate the natural degree of mobility, dietary breadth, and predation pressures nor the length of time needed for animals to reach steady state with the isotopic composition of their diet. To encompass natural diet variation, we used an independent determination of TP (TP_{SCA}) to calculate values of β and TDF in wild samples. This is done by rearranging Eq. 1:

$$\Delta\delta^{15}N_{trp-src} = (TP_{SCA} - 1) * TEF + \beta, \quad (4)$$

where $\Delta\delta^{15}N_{Trp\text{-}Src}$ is the difference in nitrogen isotopic composition of one or more trophic and source AAs. A linear fit of $\Delta\delta^{15}N$ vs. $(TP_{SCA} - 1)$ should yield a slope equal to an ecosystem level TDF_{AA} and an intercept equal to β. While there are no biological reasons linking values of β and TDF, Eq. 1 demands an empirical dependence between these values.

For each species analyzed for AA-CSIA, we used TP_{SCA} estimates from FishBase or literature citations with large sample sizes, many with direct feeding observations included in the study (Table 1). Acknowledging the inherent limitations of SCA, we accept that this method yields a reasonably close representation of TP. As no other more reliable estimates are available, we use Eq. 4 and the difference in the $\delta^{15}N$ values of trophic and source AAs, to calculate values for TDF_{AA} and β from wild marine teleosts.

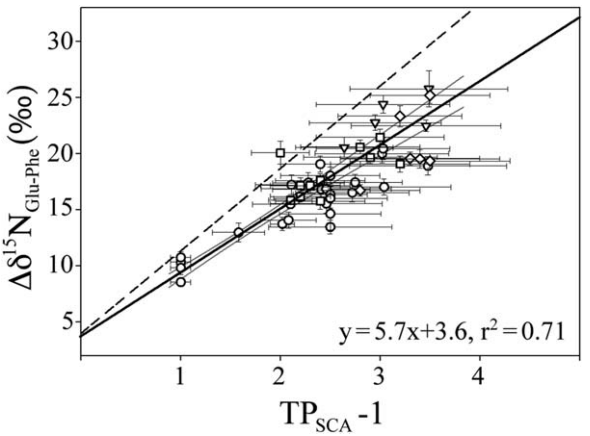

Fig. 1. Relationship between isotopic fractionation and TP in marine teleosts. Enrichment of ^{15}N between glutamic acid and phenylalanine across trophic levels, as determined by SCA, in shallow and mesophotic reef fishes (circles), deep (100–300 m) bottomfishes (inverted triangles), mesopelagic fishes (squares), and pelagic fishes (diamonds). Error bars represent one standard deviation. The line given represents a significant Deming regression ($p < 0.05$) with 95% confidence intervals as gray lines. Also plotted as a dashed line is the expected relationship from previous published studies assuming a TDF_{AA} value of 7.6‰ and β value of 3.4‰.

$\Delta\delta^{15}N$ values using Glu and Phe ($\Delta\delta^{15}N_{Glu\text{-}Phe}$) are the most commonly used in TP_{CSIA} calculations of marine organisms. Most studies have used a TDF_{AA} value for Glu and Phe of 7.6 ± 1.3‰ and β value of 3.4 ± 0.9‰ (McClelland and Montoya 2002; Chikaraishi et al. 2007, 2009, 2010; Hannides et al. 2009; Dale et al. 2011; Choy et al. 2012; McCarthy et al. 2013). In this study, a significant positive linear relationship was found between $\Delta\delta^{15}N_{Glu\text{-}Phe}$ and TP_{SCA} (Fig. 1) from which independent values for β (3.6 ± 0.5‰) and TDF_{AA} (5.7 ± 0.3‰) were fit using a linear Deming regression ($r^2 = 0.71$, df = 45, $F = 3581$ and 1994, for slope equal to 0 and 1, respectively, $p < 0.0001$). This linear relationship between $\Delta\delta^{15}N_{Glu\text{-}Phe}$ and TP_{SCA}, albeit with a higher TDF_{AA} value, was suggested by Chikaraishi et al. (2009). In an effort to test the appropriateness of the linear fit, both piecewise linear functions ($r^2 = 0.71$, df = 46, $F = 35.8$, $p < 0.0001$) and nonlinear saturating functions ($r^2 = 0.71$, df = 46, $F = 54.6$, $p < 0.0001$) were modeled with the data; neither improved relationships between $\Delta\delta^{15}N_{Glu\text{-}Phe}$ and TP_{SCA}.

It is noteworthy that the β value derived here is very similar to those from earlier studies of aquatic primary producers, especially given that this study calculated β from consumers rather than direct measurements of photoautotrophs. Additionally, our results (Fig. 1) yield a TDF_{AA} value (5.7 ± 0.3‰) similar to that previously suggested for wild elasmobranchs (4.0–5.9‰, Dale et al. 2011), harbor seals (4.3 ± 1.2‰, Germain et al. 2013), and captive fish reared on diets with low AA imbalance (5.1–5.9‰, McMahon et al. 2015b), but higher than that found in penguin blood (3.4–3.8‰, Lorrain et al.

2009) and feathers ($3.5 \pm 0.4‰$, McMahon et al. 2015a). Previous controlled feeding studies in juvenile fish (TP = 3.0) have shown a TDF_{AA} value of 7.6‰, which was consistent with results from a number of natural samples, including two organisms with TP > 3.5 (Chikaraishi et al. 2009, 2010). Data from this study, including 26 species with TP > 3.5, indicate a considerably lower TDF_{AA} value. Our results confirm that naturally derived values for TDF_{AA} can be considerably different from those in laboratory diet experiments and that a lower TDF_{AA} value of $5.7 \pm 0.3‰$ better fits wild marine teleost TP_{SCA}.

Estimation of TP using glutamic acid and phenylalanine

Using the TDF_{AA} value ($7.6 \pm 1.3‰$) calculated by Chikaraishi et al. (2009, 2010) and mean literature-derived values of β (2.7 ± 2.2), $TP_{Glu\text{-}Phe}$ resulted frequently in considerable deviation from TP_{SCA} (mean TP_{SCA}-$TP_{CSIA} = 0.70 \pm 0.44$) with lower values compared to TP_{SCA} most apparent at higher TP. An overestimate of TP_{SCA} of top predators would require that low trophic level prey is systematically overlooked (or disproportionately rapidly digested). Importantly, there has to be systematic bias in the TP_{SCA}, not just increasing error/variance with increasing TP. This is unlikely for two reasons. First, it is unlikely that low trophic level prey items are systematically overlooked given the often large numbers of stomachs typically examined for economically valuable high trophic level fishes. Second, easily digested prey represent a range of TPs, reducing the likelihood that missing them will introduce bias. For instance, gelatinous zooplankton are rapidly digested prey that will bias diet studies but they can be represented by filter feeding salps and pyrosomes or carnivorous medusa (Robison 2004) so bias in TP_{SCA} seems unlikely. Further, diet studies of pelagic predators commonly identify and quantify gelatinous salps and pyrosomes. Such diet studies include one of the pelagic predators in this study, *Lampris guttatus* (small-eye opah); nearly 10% of the diet of *L. guttatus* by weight is comprised of salps and pyrosomes (Choy et al. 2013).

It is also possible that TP_{CSIA} methods are susceptible to variation from physiological or environmental differences between species that affect the ^{15}N (and ^{13}C) enrichment in trophic relative to source AAs. The fractionation of ^{15}N and subsequent isotopic composition of tissues is ultimately affected by the synthesis, metabolism, and excretion of nitrogenous compounds. Differences in the general biochemical composition of prey items within a diet can affect catabolic and assimilation processes, as can the relative rates of metabolism and growth within an individual. The influences of these factors are difficult to separate without empirical data for each of them, such as metabolic rates or individualized stomach contents. However, gross classifications (habitat, feeding guild, and taxonomy) can be used to provide insight into the factors influencing the magnitude and variability of TDF_{AA} values.

The assumption of conservation for β and TDF_{AA} values relies upon the assumption that metabolic and catabolic processes are conserved across taxa. In this study, fish were separated into a number of groupings, distinguished by documented phylogenetic relationships (Near et al. 2012), and by known habitat and feeding guild. The underestimations of TP_{SCA} by TP_{CSIA}, assuming a TDF_{AA} value of $7.6 \pm 1.3‰$ and β value of 2.7 ± 2.2, were significantly different among several orders of fishes (Kruskal–Wallis, $p < 0.05$, Table 2). These differences may be attributed to the evolution or re-emergence of a number of factors, such as endothermy or development of alternate metabolic pathways (e.g., urea synthesis, hindgut/foregut fermentation), which could influence the $\delta^{15}N$ values of AAs in muscle tissue protein.

A commonly used method for assessment of fisheries stocks, grouping diverse fishes according to feeding guilds reflects differences in biomass flow (Austen et al. 1994) and, in the case of this study, groups of similar isotopic fractionation. It has been demonstrated that differential isotopic routing of animal and plant derived protein can alter carbon isotopic fractionation in omnivorous organisms (Kelly and Martínez del Rio 2010; McMahon et al. 2010). Consumers with a diet consisting primarily of protein may route nonessential AAs directly to tissues rather than synthesizing them from the internal carbon pool. Such routing may also limit the degree of deamination and/or transamination, thereby impacting the isotopic fractionation of nitrogen from prey to consumer.

Similarity of diet and consumer makeup results in carnivorous fish having minimal isotopic fractionation; groups of piscivores, invertivores, and planktivores, have varying fractionation factors relative to the proportion of animal protein in the diet (Martínez del Rio et al. 2009; Wolf et al. 2009). Herbivorous fish, on the other hand, are shown to have a large isotopic fractionation, associated with breakdown of a diet dissimilar to their own tissues (Mill et al. 2007; Clements et al. 2009). Here, underestimations of TP_{SCA} by AA-CSIA using Glu-Phe were smallest in the herbivores (0.12 ± 0.10) and significantly different from all other groups (Kruskal–Wallis, $p < 0.05$, Table 2) a result consistent with the recent teleost feeding experiments of McMahon et al. (2015b). TP_{CSIA} underestimated TP_{SCA} the greatest in piscivores and invertebrate predators; both are carnivorous groups with little to no plant material in the diet and, therefore, have lower fractionation values. This pattern indicates that a higher TDF_{AA} value of 7.6‰ can yield accurate TP_{CSIA} calculations for herbivores, reflecting a potential need for different TDF_{AA} values for different feeding guilds or trophic levels (e.g., Hoen et al. 2014). However, deviations between TP estimates increased linearly with mean TP of feeding guilds (Deming regression, df = 4, $r^2 = 0.96$, F_{442} and df = 4, $r^2 = 0.88$, F_{216}, for TP_{SCA} and TP_{CSIA}, respectively), revealing that a simple re-evaluation of TDF_{AA} will improve TP results.

Table 2. Mean differences in TP separated by groups. Average differences between TP estimated by stomach content analysis (TP_{SCA}) and AA compound specific isotopic analysis ($TP_{Glu\text{-}Phe}$) across taxonomic order, feeding guilds, habitat, and maturity level. Also shown in bold are significance levels of pairwise comparisons determined by a Wilcoxon rank sum test.

	TP_{SCA}–TP_{GluPhe} (SD)	*p*-value				
Order		M	L	B	P	T
Stomiiformes (S)	0.81 (0.32)	**0.00**	0.15	1.00	0.20	0.43
Myctophiformes (M)	0.38 (0.23)		0.75	**0.00**	**0.03**	0.96
Lampridiformes (L)	0.49 (0.13)			**0.04**	0.71	1.00
Beryciformes (B)	0.82 (0.29)				0.10	0.21
Perciformes (P)	0.66 (0.51)					0.75
Tetraodontiformes (T)	0.42 (—)					
Feeding guild		O	PK	I	PI	C
Herbivore (H)	0.01 (0.12)	**0.00**	**0.00**	**0.00**	**0.00**	**0.03**
Omnivore (O)	0.23 (0.13)		**0.00**	**0.00**	**0.00**	0.16
Planktivore (PK)	0.48 (0.39)			**0.00**	**0.00**	0.57
Invertebrate Predator (I)	0.67 (0.21)				**0.00**	1.00
Piscivore (PI)	0.93 (0.45)					0.41
Cleaner (C)	0.70 (0.53)					
Habitat		B	M	P		
Reef (R)	0.61 (0.44)	**0.04**	0.58	**0.00**		
Bottomfish (B)	0.36 (0.36)		0.12	**0.00**		
Mesopelagic (M)	0.50 (0.41)			**0.00**		
Pelagic (P)	1.07 (0.39)					

Groupings by habitat yielded results that again reflect the influence of metabolism, both through routing and level of activity (Table 2). Deviations between TP estimates were similar between mesopelagics (0.5 ± 0.4) and bottomfish (0.4 ± 0.4); both are known to make nightly migrations to shallower habitats for feeding (Haight et al. 1993; Meyer et al. 2007). The variations in metabolic activity during periods of migration and rest likely affect isotopic fractionation and are reflected in the similarity of TP_{SCA} underestimation.

Comparisons of neither habitat nor taxonomic grouping are independent of comparisons of feeding guilds. For example, all of the fish in the pelagic grouping were piscivores; the greatest underestimation of TP_{SCA} was seen in the pelagic group (Table 2), which also had the highest mean TP_{SCA} (4.2 ± 0.3). Furthermore, similarities in TP_{SCA} underestimation between mesopelagics and reef associated fish could be attributed to the breadth of TPs and feeding types within each group, which were larger than the remaining two habitat groupings. While a qualitative assessment, examining different categories of fish in this study begins to identify sources of variability in TP_{CSIA}. Our results converge on highlighting the influence of metabolism on isotopic fractionation, through isotopic routing of animal dietary components and the resulting enrichment associated with the breakdown of plant material in consumer guts.

Other combinations of trophic and source AAs

The difference in $\delta^{15}N$ values between nearly all combinations of individual trophic and source AAs showed clear increases with increasing TP_{SCA} (Table 3). These data were used to assess the best combinations of individual AAs for estimating TP based on both the difference in $\delta^{15}N$ values of two (one trophic, one source) AAs as well as the difference in weighted mean $\delta^{15}N$ values of groups of trophic and source AAs. For example, the coefficients of determination (r^2) for combinations involving Ser and Val were considerably lower than others, reflecting low regression strength and indicating poor predictability of the isotopic fractionation of these AAs across trophic levels. Additionally, the difference in isotopic composition of Asp and any individual source AA showed no significant relationship with TP_{SCA}.

Table 3. Significance of relationships between trophic-source fractionation and increasing trophic level. R^2 values for regressions of $\Delta\delta^{15}N_{(trophic\text{-}source)}$ vs. stomach content derived trophic level All values given result from in significant relationships ($p < 0.05$).

		Source AAs				
		Gly	**Lys**	**Phe**	**Ser**	**Thr**
Trophic AAs	Ala	0.67	0.68	0.66	0.03	0.81
	Glu	0.59	0.74	0.71	0.02	0.79
	Leu	0.62	0.69	0.67	0.03	0.80
	Pro	0.50	0.59	0.55	0.01	0.75
	Val	0.08	0.01	0.01	0.02	0.58

The weighted mean isotopic compositions of three trophic (Ala, Glu, Leu) and three source (Gly, Lys, Phe) AAs were

compared with TP_{SCA}. The use of a weighted mean ranks the importance of the isotopic analysis of AAs based on analytical uncertainty, therefore, AA $\delta^{15}N$ values with larger uncertainty (larger standard deviations) are emphasized less than those with smaller uncertainty (Hayes et al. 1990). Similar to previous publications, Glu, Ala, and Leu were included in the trophic group, and Gly, Lys, and Phe in the source group (Sherwood et al. 2005, 2011; McCarthy et al. 2007). Val and Ser were not included because of poor relationships found in preliminary regression analysis of $\Delta\delta$ values and TP_{SCA} (Table 3). Pro was also eliminated from the weighted mean for similar reasons ($\Delta\delta$ vs. TP_{SCA}, $r^2 < 0.60$), although relationships of $\Delta\delta$ values including Pro were much higher than those with Val or Ser. Although $\Delta\delta$ values between Thr and various trophic AAs showed a strong correlation with TP_{SCA}, its considerable ^{15}N depletion with each trophic step suggests that Thr is neither a trophic AA nor a reliable source AA in that it does not reflect enrichment typical of trophic step nor directly record $\delta^{15}N$ values at the base of the food web. All nine potential combinations of trophic AAs Ala, Glu, and Leu, with source AAs Gly, Lys, Phe, resulted in significant regressions with coefficients of determination greater than 0.59 (Table 3). The high correlation between $\Delta\delta$ and TP_{SCA} for these combinations led to confidence in their use as a TP indicator when using weighted mean $\delta^{15}N$ values.

Re-evaluation of β and TDF from trophic-source combinations

Beta values from previous publications show a considerable amount of variation between primary producer taxa (Table 4). Given this variability, most β values derived from teleost data using the *y*-intercept from regression analysis of $\Delta\delta_{Trp\text{-}Src}$ and ($TP_{SCA} - 1$) overlapped published values within one standard deviation (Table 4). The lowest variance in published data was found in combinations with Phe, which also had small standard deviations in data from this study. Additionally, β values derived from the intercept of the Deming regression between $\Delta\delta_{Trp\text{-}Src}$ and TP_{SCA} were similar to independently determined values for most combinations of trophic and source AAs, showing convergence of the two methods of calculating β. However, values from this study were much less variable than previously reported (*see* compilation of β values by Nielsen et al. 2015). For example, the β value derived from the isotopic analysis of primary producers using combinations of several trophic (Glu, Ala, Leu) and source (Gly, Lys, Phe) AAs was $3.3 \pm 2.5‰$ (McClelland and Montoya 2002, Chikaraishi et al. 2007, 2009, McCarthy et al. 2013), whereas the β value derived from regression analysis using Eq. 4 is in excellent agreement but with lower variability ($3.6 \pm 0.6‰$). The regression analysis averages the variability inherent in different plants and animals and is shown here to be a more accurate way of establishing a marine β value for calculating TP. This provides a method for empirically deriving a β value in areas where collection of primary producers is limited.

Table 4. β values for AA compound specific isotopic analysis (AA-CSIA). Data derived β values for the trophic-source combinations in teleosts. Standard deviations are given within parentheses. For comparison, given in italics are the calculated differences between nitrogen isotopic composition of trophic-source combinations of AAs in primary producers and standard deviations (in parentheses) based on published values from McClelland and Montoya (2002), Chikaraishi et al. (2009, 2010), and McCarthy et al. (2013).

		Source AAs				
		Gly	Lys	Phe	Ser	Thr
Trophic AAs	Ala	3.2 (0.8)	4.9 (0.7)	5.4 (0.6)	3.1 (1.9)	1.0 (1.2)
		4.4 (4.1)	*2.8 (6.2)*	*2.6 (2.3)*	*6.6 (3.0)*	*2.1 (4.7)*
	Glu	1.7 (0.9)	3.9 (0.5)	3.6 (0.5)	1.4 (1.9)	−0.1 (1.1)
		4.5 (4.1)	*2.9 (6.1)*	*2.7 (2.2)*	*6.7 (3.4)*	*2.2 (4.6)*
	Leu	1.1 (0.9)	3.0 (0.6)	2.7 (0.6)	1.4 (1.7)	−0.7 (1.2)
		2.4 (4.3)	*0.8 (5.5)*	*0.6 (3.2)*	*4.6 (3.8)*	*0.1 (4.2)*
	Pro	−0.1 (1.3)	1.0 (0.7)	−0.6 (0.9)	−3.2 (2.8)	−2.6 (1.3)
		4.4 (4.2)	*2.7 (5.7)*	*2.6 (2.3)*	*6.6 (3.1)*	*2.0 (4.9)*
	Val	−1.0 (2.0)	0.7 (2.1)	0.3 (2.0)	−38.3 (68.8)	0.9 (1.6)
		5.3 (4.8)	*3.7 (6.6)*	*3.5 (3.1)*	*7.5 (3.8)*	*3.0 (4.8)*

Table 5. TDF_{AA} values for amino acid compound specific isotopic analysis (AA-CSIA). Data derived TDF_{AA} values for the trophic-source combinations in teleosts. Standard deviations are given within parentheses.

		Source AAs				
		Gly	Lys	Phe	Ser	Thr
Trophic AAs	Ala	7.2 (0.4)	3.0 (0.4)	6.1 (0.3)	7.1 (1.2)	16.5 (0.7)
	Glu	6.8 (0.5)	5.2 (0.3)	5.7 (0.3)	6.8 (1.2)	15.8 (0.7)
	Leu	6.9 (0.5)	5.6 (0.3)	6.1 (0.3)	6.6 (1.1)	16.0 (0.7)
	Pro	6.6 (0.7)	5.5 (0.4)	6.7 (0.6)	8.1 (1.8)	15.9 (0.7)
	Val	7.9 (1.1)	6.8 (1.3)	7.3 (1.2)	32.9 (46.3)	14.9 (0.9)

TDFs derived from the regression analysis (Eq. 4) for most trophic-source combinations were all within 3‰ of one another (Table 5) with a few exceptions. Combinations with Thr had TDF_{AA} values ~10‰ larger than the remaining combinations, and at the extremes, Ala-Lys and Val-Ser were much lower and higher, respectively, than the rest. This contrasts earlier work by Chikaraishi et al. (2009), where the selection of the Glu-Phe combination was made because Glu had a significantly larger ^{15}N enrichment per TP than any other trophic AA. In fact, all combinations with Glu, data-derived TDF_{AA} values are slightly lower than with other trophic AAs. The similarity in ^{15}N enrichment of trophic AAs relative to individual source AAs aligns with the data from McCarthy et al. (2013), who found Ala, Pro, and Val to have

δ^{15}N values similar to Glx, (glutamate + glutamine). They termed these AAs "nonfractionating," reflecting isotopic steady state with the Glx pool; this coupling of AAs with Glu can be seen in our results in the convergence of TDF_{AA} values.

Previous publications have advocated the use of mean values from groups of trophic and source AAs to reduce error in TP estimations associated with analysis of only two AAs (McCarthy et al. 2007; Sherwood et al. 2011; Décima et al. 2013; Nielsen et al. 2015). The trophic fractionation associated with weighted mean trophic and source AAs, $\Delta\delta_{\bar{x}}$, had a stronger relationship with increasing TP_{SCA} than $\Delta\delta_{Glu\text{-}Phe}$ ($r^2 = 0.79$ vs. 0.71). However, the TDF_{AA} value calculated from $\Delta\delta_{\bar{x}}$ (5.7 ± 0.3‰) was identical to that from the Glu-Phe combination. These results further support the coupling of Glu with other trophic AAs and indicate that the use of $\Delta\delta_{\bar{x}}$ provides an accurate, or in some situations more accurate, calculation of TP than any combination of individual trophic and source AAs (*see* also Décima et al. 2013).

Re-evaluated TP using data-derived TDFs

TPs calculated using the data-derived values for TDF_{AA} (5.7 ± 0.3‰) and β (3.6 ± 0.5‰) for Glu and Phe more closely approximated TP_{SCA}, compared to previous estimates using a TDF_{AA} of 7.6‰ (Fig. 2a). The relationship between TP_{SCA} and $TP_{Glu\text{-}Phe}$ was similar to, but still significantly different from, a slope equal to 1 (slope = 0.920, $r^2 = 0.71$, df = 45, $F_{3.57}$, $p = 0.046$). This resulted from considerable variation in the data, resulting in a large overestimation of TP_{CSIA} of the myctophid *Benthosema glaciale* (+0.89) and an underestimation of reef-dwelling *Cephalopholis argus* (−0.80) and *Pseudocheilinus evanidus* (−0.78). These differences were, however, still smaller than the largest deviations (*C. argus* = 1.35) between TP_{SCA} and TP_{CSIA} using the previously derived TDF_{AA} (7.6‰). Furthermore, the mean absolute deviation between TP_{SCA} and $TP_{Glu\text{-}Phe}$ were smaller when using a TDF_{AA} of 5.7‰ (±0.30) than when assuming a TDF_{AA} of 7.6‰ (±0.59). The TDF_{AA} value derived from this study represents a marked improvement for calculating TP_{CSIA} of marine teleosts and is similar but slightly lower than the TDF_{AA} value for Glu and Phe of 6.6 ± 1.7‰ found in a recent compilation of 359 AA-CSIA of invertebrates, corals, bony fishes, elasmobranchs, penguins, turtles and seals from the literature (Nielsen et al. 2015).

Still greater progress was found when calculating TP using the weighted mean values of trophic AAs Ala, Glu, and Leu and source AAs Gly, Lys, and Phe, which resulted in close agreement with TP_{SCA} (slope = 0.986, $r^2 = 0.79$, df = 45, F_{8137}, $p < 0.0001$; Fig. 2b). The slope of the regression between TP estimates was not significantly different than 1 ($F_{3.57}$, $p = 0.06$). The mean absolute deviation between TP estimation methods was 0.25, an improvement from the data-derived TDF_{AA} using Glu and Phe (5.7 ± 0.3‰). Large deviations between methods were seen in *Parupeneus porphyreus* (−0.94) and again in *B. glaciale* (+0.71), although most TP_{CSIA} estimates were within 0.50 of TP_{SCA}. Overall, the use of weighted mean isotopic values of trophic AAs Ala, Glu, and Leu and source AAs Gly, Lys, and Phe resulted in a better agreement, higher coefficients of determination, and slope = 1 when compared with TP_{SCA}.

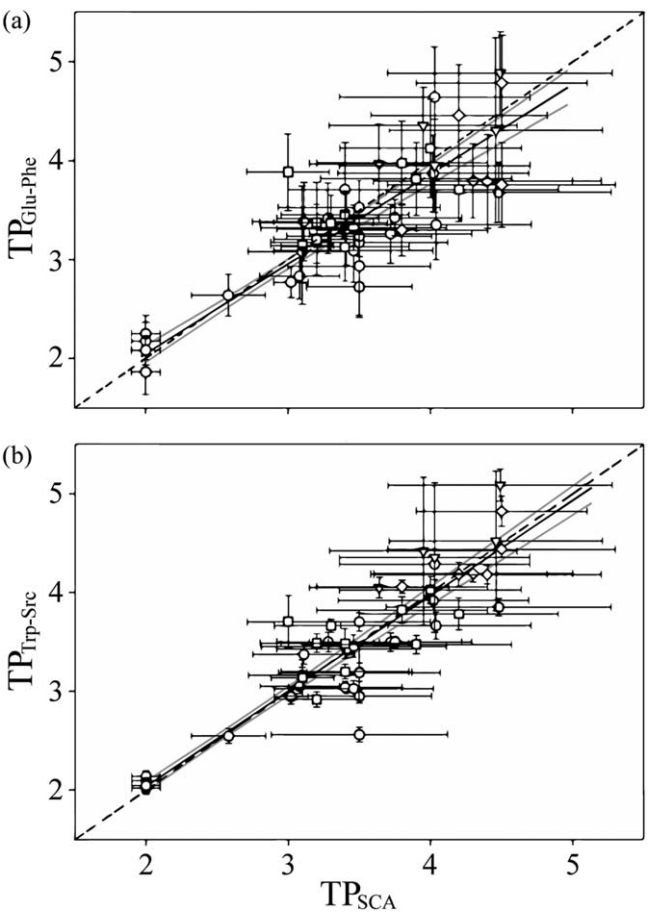

Fig. 2. Calculated TP using new TDF_{AA} and β values. TP estimated from nitrogen isotopic composition of (a) glutamic acid and phenylalanine using a $TDF_{Glu\text{-}Phe}$ of 5.7 ± 0.3‰ and β of 3.6 ± 0.5‰ and (b) a weighted mean of trophic (Ala, Glu, Leu) and source (Gly, Lys, Phe) amino acids ($TDF_{Trp\text{-}Src} = 5.7 \pm 0.3$‰, $\beta = 3.6 \pm 0.6$‰). Symbols represent different habitats, shallow and mesophotic reefs (circles), deepwater (>150 m) benthic (inverted triangles), mesopelagic depths (squares), and the pelagic open ocean (diamonds). Deming regression lines [(a) slope = 0.920, $r^2 = 0.71$, df = 45, F_{725}, $p < 0.0001$, (b) slope = 0.986, $r^2 = 0.79$, df = 45, F_{8137}, $p < 0.0001$) are given as solid lines with 95% confidence intervals as gray lines. A perfect correlation of the two methods, (slope = 1, intercept = 0) is shown as a dashed line. Error bars represent one standard deviation.

Discussion

Calculation of β from consumers

The method used in this study to derive values for β provides an independent assessment without the evaluation of

the nitrogen isotopic composition of AAs in primary producers. Interestingly, the derived values here are remarkably similar to previously measured values ($3.6 \pm 0.5‰$ vs. $2.7 \pm 2.2‰$). Previously published data were obtained from 37 samples of algae and cyanobacteria, only 9 of which were not cultured samples (McClelland and Montoya 2002; Chikaraishi et al. 2009, 2010; McCarthy et al. 2013). It is possible that more constrained culturing conditions (light, nutrients, density) could have an effect on isotopic fractionation during assimilation of nitrogen compared to more variable natural conditions. However, the resulting values from the two methods are only slightly different and the large variability in β values calculated from primary producers encompasses the value found in this study. The strong agreement of consumer-derived values with measured ones supports an alternative method for calculating β in the absence of cultured samples or in remote or otherwise difficult to sample environments.

Calculation of TDF_{AA} in teleosts

The TDF_{AA} value resulting from the combination of the isotopic composition of Glu and Phe was much lower in this study than the most commonly used value ($5.7 \pm 0.3‰$ compared to $7.6 \pm 1.3‰$). It is important to note that this value represents a community or global TDF_{AA} for marine teleosts. Discrimination factors may in fact be more variable at population levels, as evidenced by differences in TP_{CSIA} and TP_{SCA} between groups (Table 2). However, this method of deriving TDF_{AA} from wild samples provides one approach to addressing this variation. Our article is not the first to suggest that TDF_{AA} has been previously overestimated (Dale et al. 2011; Choy et al. 2012; Bradley et al. 2014), or that the biochemistry responsible for the isotopic fractionation of Glu and Phe, among others, needs further understanding (Choy et al. 2012; McCarthy et al. 2013). The data presented here indicate that published estimates for TDF_{AA} are incorrect at least for many marine teleosts. Isotopic results from this study are available through BCO-DMO (http://www.bco-dmo.org/project491309) to facilitate further comparison with AA-CSIA results from other marine telosts as they become available.

Diet impacts TDFs in a variety of ways, many of which are multifaceted factors and not easily quantified. High bulk tissue discrimination values, $\Delta\delta^{15}N$, have been measured and modeled in herbivorous fish (2.79–7.22‰), indicating that their low quality diet (high C: N ratio) and high food intake ($\sim$20% body weight d^{-1}) created a larger $\Delta\delta^{15}N$ than in carnivores (Mill et al. 2007). In contrast, low $\Delta\delta^{15}N$ values from comparison of $\delta^{15}N$ values of stomach contents and muscle tissue have been documented in situ in lower trophic level fish (planktivores: $0.80 \pm 0.9‰$, herbivores: $1.67 \pm 0.4‰$, and detritivores: $0.72 \pm 0.3‰$) compared to carnivores (all $>2.19 \pm 0.6‰$) (Wyatt et al. 2010). The recent modeling study of Nielsen et al. (2015) found that $TDF_{Glu\text{-}Phe}$ changed with feeding ecology for a wide range of marine organisms, with herbivores exhibiting the highest values and carnivores the lowest. However, AA-CSIA results on littoral mussels from the California coast found a $TDF_{Glu\text{-}Phe}$ of only 3.1‰ (Vokhshoori and McCarthy 2014) despite known feeding preferences on marine plankton with low protein contents (Vokhshoori et al. 2014). Furthermore, results from laboratory diet comparison studies have demonstrated both increased and decreased $TDF_{Glu\text{-}Phe}$ in relation to diet quality and AA imbalance between diet and consumer (Chikaraishi et al. 2015; McMahon et al. 2015b). We emphasize that results from captive laboratory feeding experiments using teleosts (McMahon et al. 2015b) and tadpoles (Chikaraishi et al. 2015) might not best represent animals living in natural environments, and study organisms with elongated guts adapted to algal diets may not adequately model predatory metabolism. Nonetheless, the lack of consensus among both wild and laboratory studies suggests that a single value describing isotopic fractionation across a range of TPs and organisms may not be realistic. However, this stands in contrast to our findings for marine teleosts based on AA-CSIA.

TPs calculated from data-derived TDFs for Glu-Phe and weighted mean trophic-source combinations were nearer to that of TP_{SCA} (Fig. 2). The method of calculating TP_{CSIA} still results in a number of over and underestimations compared to TP_{SCA}, as might be expected from two fundamentally different techniques and variability inherent in both. Anchoring TP_{CSIA} to TP_{SCA} requires several assumptions, the most obvious being that a single TP describes all individuals within a species, regardless of size/age and location. In fact, changes in diet are common in growing fish, as gape and motility limitations decrease with increasing size or age (Scharf et al. 2000; Romanuk et al. 2011). SCA is limited in its ability to capture time integrated diet, potentially missing variations due to ontogeny, prey availability, and competition. It should be noted, however, that the large sample sizes of most SCA studies partly address these complications by averaging variability. Given uncertainties in mean TP estimates from both SCA and AA-CSIA and the lack of alternative methods, estimating TP from AA-CSIA within one standard deviation of TP_{SCA} may be an acceptable goal.

The underestimation of TP_{SCA} by TP_{CSIA} calculated using a TDF_{AA} value of 7.6‰ increased with increasing TP, and this effect was also observed when comparing among feeding guilds and, to a lesser extent, habitat and taxonomic groupings. Our data indicate that phylogeny does not impact isotopic fractionation at least above the ordinal level. Significant differences between two of the three most ancient orders of fish in this study, Stomiiformes and Myctophiformes (Table 2) can be explained by feeding ecology, with the former piscivorous and the latter zooplanktivorous, impacting isotopic fractionation and subsequent calculations of TP from isotopic data. We can infer from our results that TP_{CSIA} may be influenced by the amount of plant and animal protein in the diet, and in that way by trophic level

itself, as the proportion of plant material consumed typically decreases with increasing trophic step.

The TDF_{AA} values we present in this article, derived from wild samples, provide an alternative way to calculate TP using CSIA, compared to controlled feeding studies. Propagated errors associated with TP_{CSIA}, as described in Eq. 4, were all less than 0.5, indicating the power of this technique in its precision. We have found that the calculation of TP from a weighted mean $\delta^{15}N$ values of multiple trophic and source AAs resolved much of the discrepancy between TP_{CSIA} and TP_{SCA}. The required data for this approach is generated as part of standard AA-CSIA protocols, including triplicate analysis of each sample, and we suggest that such averages be adopted for teleost TP estimation in future studies.

TDFs for the atypical AA threonine

The most significant linear correlation between $\Delta\delta^{15}N$ values and TP was found for threonine regardless of trophic AA used. Although the $\delta^{15}N$ values of threonine were quite different from all other AAs ($\delta^{15}N$ values as low as $-30‰$ compared to $-5‰$ in source AAs and $+9‰$ in trophic AAs) the magnitude of ^{15}N depletion of threonine with increasing TP is constant, suggesting a possible importance in calculating TP. Threonine has been previously disregarded due to an inability to quantify the $\delta^{15}N$ value (Chikaraishi et al. 2009) or its seemingly "outlier" negative $\delta^{15}N$ values (Sherwood et al. 2011), despite earlier work which found improved trophic transfer estimates when it was included in average calculated source values for plankton and particulate matter (McCarthy et al. 2007).

Interestingly, both Phe and Thr stood out in an evaluation of $\delta^{15}N$ values of AAs for identifying prokaryotic and eukaryotic sources in food webs (McCarthy et al. 2013). Phe has been considered as a model source AA, as the major metabolic route of Phe does not involve either breakdown or formation of nitrogen bonds (Chikaraishi et al. 2007; Ohkouchi et al. 2015). In contrast, considerable ^{15}N depletion ($>6‰$) has been measured with each trophic transfer of Thr (Hare et al. 1991; Styring et al. 2010). Although this ^{15}N depletion with each trophic transfer indicates that Thr does not encode directly the $\delta^{15}N$ values of primary producers, our results indicate that the magnitude of ^{15}N depletion with each trophic transfer is consistent, evident by the strong regressions seen in this study. It is possible that change in the $\delta^{15}N$ value of Thr with each trophic transfer is so great that variability due to changes in metabolic pathways appears less significant. However, before it can be used with confidence, an understanding of what metabolic processes control the $\delta^{15}N$ value of Thr with TP is required.

Comments and recommendations

Results of this study provide valuable insights to the application of AA-CSIA to trophic studies of marine teleosts. Finding a unique combination of trophic and source AAs which accurately predicts TP across taxa is a complex task. For teleosts, evaluation of TP from differences in the $\delta^{15}N$ values of Glu and Phe from the 47 fish species in this study indicates a TDF_{AA} value significantly lower than the most commonly used value ($5.7 \pm 0.3‰$ compared to $7.6 \pm 1.3‰$). Still, inaccuracies in TP were seen in certain species.

Alternatively, the weighted mean $\delta^{15}N$ values of multiple trophic (Ala, Glu, and Leu) and source (Gly, Lys, and Phe) AAs provided a reliable regression for deriving TDF_{AA}. TP estimates using the difference in weighted mean $\delta^{15}N$ values for multiple trophic and source AAs showed increased consistency with TP_{SCA}. While reducing some of the problems associated with using single AA combinations, such as the commonly used pairing of Glu and Phe, the weighted mean approach still allows for comparison of baseline values using canonical source AA $\delta^{15}N$ values. Therefore, the use of weighted mean $\delta^{15}N$ values of the multiple trophic and source AAs analyzed in at least triplicate may stand as the most useful combination for trophic studies from AA-CSIA.

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