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Amino acid ^{15}N trophic enrichment factors of four large carnivorous fishes

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ABSTRACT

Ecosystem-based fisheries management strategies require knowledge of trophic relationships. Trophic position (TP) estimates from compound specific nitrogen isotopic analysis of amino acids (AA-CSIA) show promise as the method can disentangle confounding factors associated with changing $\delta^{15}N$ values at the base of the food web, but it has yet to be tested in many organisms. This novel technique requires two empirically determined biological parameters: 1) β, the difference in $\delta^{15}N$ values between glutamic acid (glu) and phenylalanine (phe) in primary producers and 2) trophic enrichment factor (TEF), the ^{15}N enrichment of glu and phe at each trophic step. Values of β (3.4‰) and TEF (7.6‰) have been suggested for animals in aquatic environments; however recent observations indicate that TEF values may be variable, particularly among elasmobranchs where urea retention may alter nitrogen isotope fractionation between glu and phe. To test these uncertainties, we determined TEF values for three species of sharks, sand tiger (*Carcharias taurus*), lemon (*Negaprion brevirostris*), and leopard sharks (*Triakis semifasciata*), and one teleost species, opakapaka (*Pristipomoides filamentosus*) grown on controlled and well characterized diets for durations ranging from three (*T. semifasciata*) to over five years (*P. filamentosus*). TEF values for both elasmobranchs and opakapaka were ~2‰, significantly lower than TEFs previously reported. These results do not support the hypothesis that urea retention lowers ^{15}N trophic enrichment between glu and phe in elasmobranchs. Rather, isotopic enrichment factors may be primarily driven by differences in dietary protein quality, leading to distinct TEFs for herbivores (~7.6‰) and carnivores (<7.6‰). We propose a method to calculate TP which integrates different TEF values for herbivores and carnivores.

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1. Introduction

Successful fisheries management strategies rely on understanding ecological linkages with methods such as trophic models. This technique requires accurate estimates of trophic positions (TP) to establish an organism's role in its environment and evaluate potential anthropogenic effects on ecosystem dynamics (Branch et al., 2010). However, measuring an organism's TP can be challenging. Traditional methods of diet studies such as stomach content analyses and bulk tissue isotope analyses suffer from a number of biases and uncertainties. Stomach content analyses measure what was ingested but not necessarily integrated into a consumer's tissues. This method requires large sample sizes, represents only an organism's most recent meal, and may be biased due to stomach eversion upon capture (DeMartini et al., 1996). Although bulk tissue or organism isotope analyses provide temporally and spatially integrated insight to diet and habitat, variability in trophic discrimination factors and source nitrogen $\delta^{15}N$ values can complicate ecological interpretations (Chikaraishi et al., 2009; Post, 2002).

Compound-specific isotopic analysis of amino acids (AA-CSIA) offers an integrated, relatively unbiased evaluation of an organism's trophic biology and has the potential to considerably advance food web studies. Certain amino acids (AAs), termed "source" AAs, do not become significantly ^{15}N-enriched in consumer tissues relative to their source, while $\delta^{15}N$ values for "trophic" AAs (*sensu* Popp et al., 2007) are highly enriched in ^{15}N with each trophic transfer (McClelland and Montoya, 2002). AA-CSIA differs from bulk tissue analysis because a consumer sample contains source and trophic AAs. These $\delta^{15}N$ values determine the consumer's TP and isotopic composition of primary producers. (e.g., Chikaraishi et al., 2007; Olson et al., 2010; Popp et al., 2007; Sherwood et al., 2011). Chikaraishi et al. (2007) suggest that the differences in isotopic fractionation between source and trophic AAs arise

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from differing dominant metabolic pathways that affect the degree of amino acid deamination and transamination, which cleave C – N bonds and result in isotopic fractionation.

TP estimates are predominantly made with the nitrogen isotopic compositions of trophic AA glutamic acid ($\delta^{15}N_{glu}$) and source AA phenylalanine ($\delta^{15}N_{phe}$):

$$TP = \frac{\left(\delta^{15}N_{glu} - \delta^{15}N_{phe} - \beta\right)}{TEF} + 1 \quad (1)$$

where TEF is the trophic enrichment factor (the ^{15}N enrichment of glutamic acid relative to phenylalanine at each trophic step) and β is the difference between $\delta^{15}N_{glu}$ and $\delta^{15}N_{phe}$ values in primary producers (Fig. 1).

In order to broadly apply AA-CSIA in trophic studies, β and TEF must be constrained across a broad range of taxa and physiologies. In controlled feeding experiments, Chikaraishi et al. (2009) determined β values for 17 photoautotrophs and TEF values for 4 consumers, including zooplankton (TP = 2) and juvenile fish *Sebastes schlegi* and *Paralichthys olivaceus* (TP = 3). Their results indicated little variability in β (3.4‰) and TEF (7.6‰), suggesting that these values may be universal (Chikaraishi et al., 2009, 2010) (Fig. 1A). More recent results indicate potential variability in β as a function of dietary nutrients and metabolic physiology (McCarthy et al., 2013; Vander Zanden et al., 2013). Further, lower TEF values have been found for a number of carnivorous animals including penguins, sharks, and seals (Dale et al., 2011; Decima et al., 2013; Germain et al., 2013; Lorrain et al., 2009).

A TEF of 7.6‰ was determined from a limited breadth of samples at low TPs and may not be representative of fully mature or higher TP fishes (Fig. 1B). In addition, a recent study with elasmobranchs questioned 7.6‰ as a universal TEF value (Dale et al., 2011). Unlike marine teleost fishes, most elasmobranchs are isosmotic or slightly hyperosmotic and retain urea, $(NH_2)_2CO$, for osmoregulation. The urea nitrogen in elasmobranchs is derived from the amide nitrogen of glutamine (Julsrud et al., 1998). It has been hypothesized that the retention of ^{15}N-depleted urea may "mask" trophic ^{15}N enrichment in bulk analysis of elasmobranch tissues (Fisk et al., 2002; Hussey et al., 2010; Kim and Koch, 2012; Logan and Lutcavage, 2010). Additionally, Dale et al. (2011) suggested that hepatic urea production in elasmobranchs could result in lower ^{15}N trophic enrichment in glutamic acid. To date, no AA-CSIA data exist for experimental feeding studies of juvenile to adult marine carnivorous fishes or elasmobranchs.

We determined TEF values for a variety of trophic-source AA combinations for higher TP consumers – three elasmobranch species (sand tigers *Carcharias taurus*, lemon *Negaprion brevirostris*, and leopard *Triakis semifasciata* sharks) as well as one teleost species (opakapaka *Pristipomoides filamentosus*) – grown on controlled and well-characterized diets. Due to urea retention and carnivorous diets, we hypothesized that the TEF value for elasmobranchs would be less than 7.6‰. Furthermore, we reasoned the TEF value for opakapaka, a large carnivorous teleost, would also be less than 7.6‰, but greater than the sharks' TEF value since teleosts neither produce nor retain urea.

2. Materials and methods

2.1. Study animals

The nitrogen isotopic composition of individual AAs in 13 muscle tissue samples represented 4 consumer species (three sand tiger sharks, three leopard sharks, one lemon shark, and six opakapaka) and 24 diet organisms (including anchovy, haddock, trevally, saithe, mackerel, whiting, mullet, octopus, krill, and squid). The sand tiger and lemon sharks sampled were caught in the wild, maintained in aquaria for at least 6 years, and euthanized due to medical conditions. These medical issues did not likely affect the feeding behavior or overall condition of the sharks (Hussey et al., 2010). Detailed results of bulk isotope analysis of the sharks and their feed are available (Hussey et al., 2010). All sharks were mature with the exception of the lemon shark (Table 1).

The leopard sharks sampled were kept in captivity at the Long Marine Laboratory (Univ. of California, Santa Cruz) and fed a constant diet of squid from Monterey Bay for over three years to ensure that they had reached a steady state with their dietary nitrogen isotopic composition (Table 1). Detailed results of bulk tissue isotope analysis of these sharks can be found in Kim et al. (2012).

A brood stock of opakapaka have been kept in captivity at Hawai'i Institute of Marine Biology and fed a relatively constant diet for ~5 years. In July 2009, one opakapaka, caught as a very small juvenile (7–10 cm) in 1999, died from net entanglement. Between May 25 and June 1, 2011, five opakapaka, raised in captivity from eggs, died from net entanglement after the entire brood stock was transferred to another cage. The six individuals were mature and fatty with high C:N ratios (Table 1). We analyzed the white muscle tissue from the six opakapaka as well as most recent (6 months) samples of their diet of anchovies, squid, and krill. White muscle samples were freeze-dried, homogenized, and bulk isotopic composition determined prior to AA-CSIA.

2.2. Bulk tissue isotopic analysis

Scales and skin were removed and white muscle tissue was dissected from each opakapaka specimen. Samples were freeze-dried for ~48 h

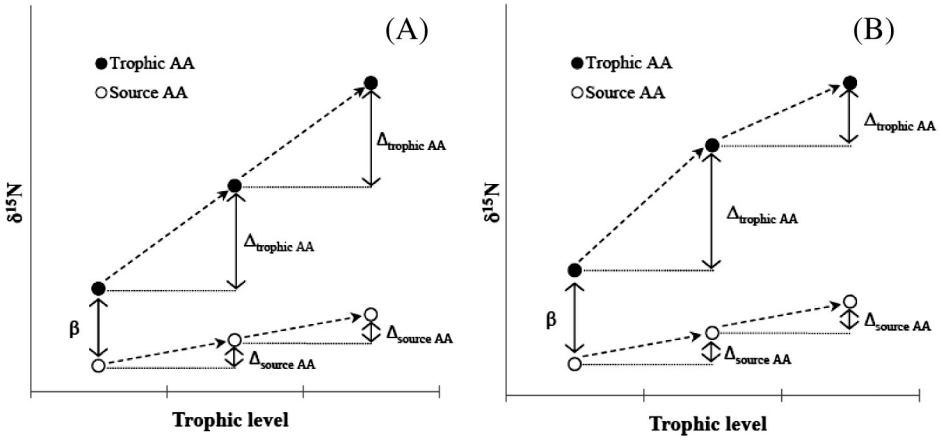

Fig. 1. Proposed relationships between nitrogen isotopic composition of AAs and trophic position. β represents the $\delta^{15}N$ difference between trophic (closed symbols) and source (open symbols) AAs in primary producers. Δ represents the ^{15}N enrichment of AAs in a trophic system where the TEF is constant (A) and where TEF decreases with TP (B). Adapted from Chikaraishi et al. (2009).

Table 1
Details of consumers from this study. Lengths for sharks (opakapaka) are measured as total (fork) lengths.

Location	Common name	L (cm)	Sex	Maturity	Estimated age (yr)	$\delta^{15}N$ (‰)	$\delta^{13}C$ (‰)	C:N	$\Delta^{15}N_{Bulk}$ (‰)
DSW[a]	Sand tiger	198	M	Mature-adult	7–8	15.0	−17.1	2.7	2.27
TD[a]	Sand tiger	242	F	Mature-adult	10–11	15.6	−16.3	2.7	2.15
BP[a]	Sand tiger	261	M	Mature-adult	12–13	16.3	−16.3	2.7	2.14
BP[a]	Lemon shark	199	M	Sub-adult	9–10	12.9	−17.0	2.7	2.60
LML[a]	Leopard shark	91	M	Juvenile	7	17.0	−16.8	2.7	3.70
LML	Leopard shark	82	F	Juvenile	5	16.9	−16.8	2.5	3.60
LML	Leopard shark	81	F	Juvenile	5	17.3	−16.7	2.6	4.00
HIMB[a]	Opakapaka	57	U[b]	Mature	>10	15.8	−18.6	5.4	2.94
HIMB	Opakapaka	45	U	Mature	~5	15.3	−16.4	3.4	2.44
HIMB	Opakapaka	47	U	Mature	~5	16.7	−15.5	3.4	3.84
HIMB	Opakapaka	41	U	Mature	~5	15.1	−17.3	3.9	2.24
HIMB	Opakapaka	41	U	Mature	~5	15.1	−16.2	3.4	2.24
HIMB	Opakapaka	44	U	Mature	~5	15.9	−15.7	3.3	3.04

Hussey et al., 2010 and Kim et al., 2012.
[a] Deep Sea World (DSW), The Deep (TD), and Blue Planet (BP) aquaria, Long Marine Lab (LML), and Hawaii Institute of Marine Biology (HIMB).
[b] Unknown.

and then ground and homogenized with a mortar and pestle. Homogenized tissues were split; each portion was weighed and packaged into either tin capsules for bulk tissue isotopic analysis (0.4–0.5 mg) or combusted glass reaction vials for AA-CSIA (~5 mg).

Bulk tissue $\delta^{13}C$ $\delta^{15}N$ values were determined using an isotope ratio mass spectrometer (Delta^{Plus}XP) coupled to an elemental analyzer (Costech ECS 4010/ConFlo IV). Isotopic values are reported in conventional δ-notation relative to international standards atmospheric N_2 and V-PDB for N and C, respectively. Accuracy and precision of $\delta^{13}C$ and $\delta^{15}N$ values were <0.2‰ based on two well characterized, in-house reference materials (glycine and homogenized tuna muscle). To ensure that the nitrogen isotopic composition of the opakapaka diet remained relatively constant, the bulk isotopic composition of two subsets of feed samples spanning ~6 months was determined.

2.3. Preparation for compound specific amino acid analysis

All muscle tissue samples (shark, opakapaka, and diet organisms) were prepared for AA-CSIA by hydrolysis, then subsequent esterification and trifluoroacetylation according to the method described by Hannides et al. (2009). Prior to AA-CSIA, samples were evaporated to dryness, redissolved in 100 μL ethyl acetate and analyzed within 24 h. Occasionally, when left in ethyl acetate over 24 h before analysis, samples had to be rederivatized. In this case, samples were dried and 0.5 mL TFAA and 0.5 mL ethyl acetate were added. The solution was allowed to stand at room temperature for 1 h after which the sample was dried, 100 μL ethyl acetate was added, and samples were immediately analyzed.

2.4. Compound specific stable nitrogen isotope analysis

The $\delta^{15}N$ values of individual amino acids were measured using isotope ratio monitoring gas chromatography–mass spectrometry (Delta V Plus/Trace GC/GC-C III Interface). All samples were analyzed at least in triplicate, and the measured N isotopic compositions were normalized to known $\delta^{15}N$ values of two internal reference compounds (norleucine and amino adipic acid) co-injected with each sample. The reproducibility of isotopic analysis of glutamic acid and phenylalanine averaged ±0.3‰ and ±0.5‰ (1 standard deviation [S.D.]) respectively and ranged from ±0.1‰ to ±0.8‰ for glutamic acid and ±0.1‰ to ±1.2‰ for phenylalanine. Accuracy of the isotopic analysis was estimated using the known $\delta^{15}N$ value for norleucine to determine a measured $\delta^{15}N$ value of amino adipic acid, treating it as an unknown in all samples. Accuracy of these internal reference amino acids averaged ±0.4‰ (1 S.D.) and never exceeded ±0.7‰.

2.5. Trophic enrichment factor calculations

TEF values were calculated using an isotope mass balance approach similar to Hussey et al. (2010), but the $\delta^{15}N$ values of trophic and source AAs were also considered (Eq. (2)),

$$TEF = \left(\delta^{15}N_{tc} - \delta^{15}N_{td}\right) - \left(\delta^{15}N_{sc} - \delta^{15}N_{sd}\right) \quad (2)$$

where $(\delta^{15}N_{tc} - \delta^{15}N_{td})$ is the nitrogen isotopic difference between the trophic AA of the consumer ($\delta^{15}N_{tc}$) and diet ($\delta^{15}N_{td}$) while $(\delta^{15}N_{sc} - \delta^{15}N_{sd})$ is the difference in the source AA between consumer ($\delta^{15}N_{sc}$) and diet ($\delta^{15}N_{sp}$). Averaged $\delta^{15}N$ values for source and trophic AAs of diet were calculated with Eq. (3):

$$\delta^{15}N_d = \sum_n w_1\left(\delta^{15}N_{d_1}\right) + w_2\left(\delta^{15}N_{d_2}\right) + \ldots + w_n\left(\delta^{15}N_{d_n}\right) \quad (3)$$

where $\delta^{15}N_{d_n}$ represents the $\delta^{15}N$ value of diet organism n and where w_n is the fraction of nitrogen contributed to the consumer by diet organism n. Mean $\delta^{15}N$ values were weighted based on analytical reproducibility. TEF values for the sharks were pooled and compared with TEF values obtained for opakapaka and zooplankton, *S. schlegi*, and *P. olivaceus* (Chikaraishi et al., 2009, 2010) using a Kruskal–Wallis rank sum test. Errors were propagated through the equations, with more weight given to values with lower uncertainty. To determine which groups were significantly different, relationships were further evaluated using a post-hoc non-parametric multiple comparison procedure ($\alpha = 0.05$) using MATLAB version R2012a.

3. Results

3.1. Bulk isotope analysis

There was little change in nitrogen isotopic composition of opakapaka diet as determined from the standard deviations for averaged (±S.D.) feed values, which were less than 0.5‰ ($\delta^{15}N_{anchovy}$: 14.1 ± 0.4‰, $\delta^{15}N_{squid}$: 14.0 ± 0.5‰, $\delta^{15}N_{krill}$: 6.0 ± 0.4‰). Opakapaka bulk $\delta^{15}N$ values ranged from 15.1‰ to 16.7‰ (Table 1). Bulk $\delta^{13}C$ values were considerably more variable, but inversely correlated to C:N ratios by mass ($y = -1.30x - 11.65$; $R^2 = 0.91$; $P < 0.005$). Opaka 1 and 4 had C:N ratios greater than 3.5, above the recommended value for application of $\delta^{13}C$ values to food web studies (Post et al., 2007). High C:N ratios coupled with low $\delta^{13}C$ values indicate that these fish had high lipid content, however high C:N ratios measured should not affect nitrogen isotopic composition as lipids do not contain nitrogen. Opakapaka were enriched in ^{15}N relative to their diet by 2.8 ± 0.6‰ (Table 1). Elasmobranch bulk ^{15}N discrimination factors (Table 1)

ranged from 2.2 ± 0.1‰ (sand tiger sharks; Hussey et al., 2010) to 3.7 ± 0.4‰ (leopard sharks; Kim et al., 2012).

3.2. Compound specific isotope analysis of amino acids

Across all consumer and diet samples the following AAs were consistently detected and their $\delta^{15}N$ values measured: alanine, glycine, serine, leucine, proline, aspartic acid, glutamic acid, phenylalanine, and lysine (Tables 2, 3). Multiple samples of some diet organisms were analyzed to ensure isotopic consistency (Table 2). Analyzed diet organisms for the BP lemon shark represented 90% of the shark's diet.

$\delta^{15}N$ differences between consumers and diet (Δ) were similar for glutamic acid, phenylalanine, aspartic acid, and lysine among all consumers in this study, while a few other AAs had marked departures (Fig. 2, also compare Tables 2 and 3). Alanine was enriched in ^{15}N by >4‰ in all opakapaka relative to diet while the ^{15}N-enrichment in sharks relative to diet ranged from −0.4‰ to 4.0‰. Glycine Δ values in sharks and their diet were variable (−6.8‰ to 4.9‰), but were generally greater than for opakapaka (0.4 ± 0.3‰). Serine was depleted in ^{15}N across all consumers relative to diet, with smaller differences among opakapaka than sharks. The $\delta^{15}N$ difference of leucine in opakapaka and their diet was larger (5.9 ± 0.2‰) than sharks (3.0 ± 0.2‰). The ^{15}N enrichment in lysine in opakapaka relative to feed (0.6 ± 0.2‰) was less than that found in sharks (1.9 ± 0.1‰).

Shark and opakapaka Δ values for valine, leucine, and proline were similar to those found for zooplankton and zooplanktivorous fish (*S. schlegi*, and *P. olivaceus*) (Chikaraishi et al., 2009) (Fig. 3). Glutamic acid Δ values in the sharks and opakapaka were lower than those reported in zooplankton and juvenile zooplanktivorous fish (Chikaraishi et al., 2009). In contrast, Δ values for alanine in opakapaka (5.7 ± 0.3‰) agreed with the previously reported value (6.0 ± 1.9‰), while sharks were considerably lower (1.1 ± 0.2‰). Phenylalanine was slightly enriched in opakapaka (1.2 ± 0.2‰) and sharks (leopard sharks: 0.9 ± 0.1‰, sand tiger sharks: 1.3 ± 0.3‰, lemon shark: 2.2 ± 0.8‰) relative to diet compared with zooplankton and juvenile zooplanktivorous fish (0.4 ± 0.4‰) (Chikaraishi et al., 2009).

3.3. Trophic enrichment factors

Multiple trophic-source AA combinations were considered when calculating TEF values. Phenylalanine was chosen as the source AA (see Discussion section). Five trophic AAs were examined: glutamic acid, alanine, valine, leucine, and proline. A Kruskal–Wallis test revealed significant consumer group differences in TEF values from all trophic-source AA combinations except in the valine–phenylalanine ($TEF_{val-phe}$) grouping (Table 4). A post-hoc non-parametric multiple comparison procedure indicated that the $TEF_{glu-phe}$ values for sharks and opakapaka were significantly lower than those reported for zooplankton and juvenile zooplanktivorous fish (Chikaraishi et al., 2009). $TEF_{ala-phe}$ values of zooplankton, juvenile zooplanktivorous fish, and sharks were significantly different. $TEF_{leu-phe}$ values of shark species were variable (sand tigers: 0.2 ± 0.5‰; lemon shark: 0.5 ± 1.1‰; leopard sharks: 2.9 ± 0.4‰) but when considered as a group, the $TEF_{leu-phe}$ for sharks was significantly lower than the opakapaka or previous studies (Chikaraishi et al., 2009). Previous estimates of $TEF_{pro-phe}$ values were significantly greater than those found in this study (Chikaraishi et al., 2009).

We also evaluated TP calculations using a weighted average of trophic amino acid combinations to calculate TEF and β values. Trophic AA combinations included alanine–valine–leucine–proline (AVLP) and valine–leucine–proline (VLP). These AA combinations were chosen because they were consistently detected and previously used as trophic

Table 2
Average AA nitrogen isotopic composition of feed samples. Amino acids with concentrations below measurement capabilities are not listed.

Identification	n	% diet	$\delta^{15}N$ (‰, relative to air)										
			Ala	Gly	Thr	Ser	Val	Leu	Pro	Asp	Glu	Phe	Lys
Opakapaka													
Anchovy	2	42.9	26.5	8.9	−1.1	9.8	19.7	20.7	19.7	21.3	23.2	8.9	11.9
Squid	1	42.9	27.5	2.3	−9.8	10.8	23.1	24.8	26.8	19.7	25.3	7.3	3.7
Krill	1	14.3	18.7	0.6	−6.2	3.0	13.1	12.4	12.4	14.6	16.3	4.8	5.6
$\bar{x}$			25.8	4.9	−5.6	9.2	20.2	21.3	21.7	19.7	23.1	7.6	7.4
TD sand tiger													
Haddock	2	82.8	27.1	5.7	−15.1	9.8	25.3	24.6	19.4	24.3	24.2	2.4	3.5
Trevally	1	17.2	24.1	3.2	−9.0	6.7	20.3	22.5	21.3	17.1	23.4	5.6	7.8
$\bar{x}$			26.6	5.3	−14.0	9.3	24.4	24.2	19.7	23.1	24.1	3.0	4.3
DSW sand tiger													
Trevally	2	43.0	26.6	5.8	−13.0	6.9	23.7	23.2	22.7	19.0	25.0	5.3	6.6
Saithe	1	33.7	25.6	n.d.[a]	−13.6	2.5	19.9	22.1	23.0	23.4	23.4	3.2	4.6
Mackerel	1	23.4	24.3	−0.6	−11.4	6.8	22.5	21.6	21.6	21.2	23.6	3.3	5.9
$\bar{x}$			25.7	n.d.	−12.8	5.4	22.1	22.5	22.5	21.0	24.1	4.1	5.8
BP sand tiger													
Trevally	2	98.1	27.3	4.6	−11.0	7.5	26.0	25.7	22.5	19.0	26.1	8.1	5.6
Whiting	1	1.2	29.6	9.0	−10.9	10.0	25.5	25.4	23.0	27.3	26.7	8.0	7.6
Mullet	1	0.7	27.4	11.6	3.7	9.2	21.1	19.5	13.7	22.8	20.4	8.6	9.0
$\bar{x}$			27.3	4.7	−10.9	7.5	26.0	25.6	22.5	19.1	26.1	8.1	5.6
BP lemon shark													
Octopus	2	80.7	22.3	5.2	−16.9	8.1	21.4	20.7	19.4	16.5	21.2	4.2	5.8
Squid	1	9.7	27.2	−1.8	−18.3	12.0	26.5	24.0	27.4	17.9	25.1	3.8	5.3
Giant squid	1	9.7	30.1	0.7	−18.4	10.1	25.5	26.4	27.1	19.9	26.6	5.8	5.7
$\bar{x}$[b]			23.8	4.1	−17.3	8.8	22.5	21.8	21.2	17.1	22.4	4.4	5.8
Leopard sharks													
Squid	5	100	26.2	7.9	−12.1	13.2	20.1	22.5	22.9	17.1	22.3	6.3	6.9

Abbreviations: alanine (Ala), glycine (Gly), threonine (Thr), serine (Ser), valine (Val), leucine (Leu), proline (Pro), aspartic acid (Asp), glutamic acid (Glu), phenylalanine (Phe), lysine (Lys).

[a] n.d., no data.

[b] Percent diet of the lemon shark was normalized to 100% for $\bar{x}$ calculations.

Table 3
Average AA nitrogen isotopic composition of consumers. Amino acids with concentrations below measurement capabilities are not listed.

Identification	δ^{15}N (‰)											
	Ala	Gly	Thr	Ser	Val	Leu	Pro	Asp	Glu	Phe	Tyr	Lys
Elasmobranchs												
TD sand tiger	29.3	11.5	−21.0	8.7	28.4	26.5	23.2	24.8	28.1	6.0	n.d.	6.5
DSW sand tiger	26.6	4.2	−19.2	4.6	25.7	24.6	26.5	21.9	27.3	5.8	n.d.	6.6
BP sand tiger	26.9	9.6	−14.3	8.7	25.7	24.3	25.1	18.3	26.5	6.0	n.d.	6.4
BP lemon shark	24.5	−2.8	−22.8	4.6	n.d.	24.6	21.4	18.2	25.9	6.6	n.d.	6.1
FL leopard shark	30.2	10.1	−12.9	7.2	n.d.	28.0	28.2	21.4	27.3	9.1	n.d.	9.1
CS leopard shark	26.7	9.3	−17.3	7.0	23.2	26.5	26.1	19.0	25.7	7.0	14.5	9.3
FS leopard shark	n.d.	9.2	−17.5	7.9	n.d.	25.7	29.1	19.5	26.2	7.2	13.9	6.9
Opakapaka												
Opaka 1	n.d.	8.3	n.d.	9.9	n.d.	26.5	25.9	21.5	25.6	8.4	10.3	7.3
Opaka 2	30.1	3.6	−13.6	8.3	24.5	26.8	25.5	22.2	25.1	9.2	10.0	7.6
Opaka 3	32.3	5.8	n.d.	8.0	n.d.	28.1	26.0	22.9	27.8	8.6	n.d.	8.2
Opaka 4	32.2	5.0	n.d.	8.4	n.d.	26.8	26.0	21.5	24.9	8.1	11.4	8.2
Opaka 5	31.7	4.3	−15.6	7.4	n.d.	26.9	25.2	21.8	25.1	8.5	8.6	n.d.
Opaka 6	30.8	3.9	−16.8	6.8	25.7	27.8	25.6	22.4	26.9	9.2	10.7	8.3

AAs (Chikaraishi et al., 2009, 2010). TEF values were calculated for sharks, opakapaka, zooplankton, and juvenile zooplanktivorous fish (Chikaraishi et al., 2009) using Eq. (2) with the averaged ^{15}N enrichment of the trophic AA combinations relative to source AA (Fig. 4). While both opakapaka and shark TEF values were lower than those reported for zooplankton and juvenile zooplanktivorous fish (Chikaraishi et al., 2009), only shark TEF combinations were significantly lower (Table 4). Shark TEF_{AVLP} values were lower than TEF_{VLP}, as alanine was scarcely enriched in ^{15}N relative to feed (Fig. 2, Table 4).

4. Discussion

4.1. Trophic enrichment factors and bulk isotope discrimination

Bulk tissue discrimination factors generally followed similar patterns as AA TEF values. Sand tigers tended to have the lowest bulk discrimination and $TEF_{glu-phe}$ (Table 1). Opakapaka had slightly larger discrimination factors than sand tigers that agreed with previously reported teleost values (Caut et al., 2009; Vanderklift and Ponsard, 2003). Leopard sharks consistently exhibited higher discrimination factors and $TEF_{glu-phe}$.

Three source AAs were considered when calculating TEF values: glycine, serine, and phenylalanine. An ideal source AA would have little ^{15}N enrichment relative to diet and low variability, and be consistently present for isotopic analysis. Phenylalanine ^{15}N enrichment relative to diet (Δ) consistently remained low across all samples and exhibited the lowest variability, making it the most appropriate source AA, in agreement with previous findings (Chikaraishi et al., 2009). In comparison, a preferred trophic AA for TP estimates must also be consistent and present in measureable quantities. Glutamic acid Δ values were among the most consistent of all trophic AAs and this AA was also consistently present in measurable concentrations in all organisms studied, supporting its use as a preferred trophic AA for TP estimates. Glutamic acid Δ for opakapaka (3.8 ± 0.1‰) and sharks (2.8 ± 0.1‰) was however much lower than those found for zooplankton and juvenile zooplanktivorous fish (*S. schlegi* and *P. olivaceus*) (8.0 ± 1.1‰; Chikaraishi et al., 2009) (Fig. 4). Our results suggest a lower $TEF_{glu-phe}$ than 7.6‰ for sharks and opakapaka and potentially other elasmobranchs and carnivorous teleost fishes (Table 4).

Recently, TP estimates from AA-CSIA rely on weighted averages of trophic amino acid combinations. TPs calculated using weighted TEF values are potentially more accurate and less susceptible to individual AA isotopic variability (e.g., Decima et al., 2013; Sherwood et al., 2011). Similar to our other trends, the use of average trophic AA δ^{15}N values resulted in lower TEF values for sharks and opakapaka in this study than for zooplankton and juvenile zooplanktivorous fish (Chikaraishi et al., 2009). Shark TEF values for the AVLP AA combination were much lower than those found in opakapaka, mainly a result of little ^{15}N enrichment in alanine between sharks and feed. When alanine was not considered (VLP), shark and opakapaka TEF values agreed more closely.

Similar TEF values for sharks and opakapaka suggest similar fractionation processes and do not support the hypothesis that shark TEF values are low due to urea retention. Differences in fractionation due to a

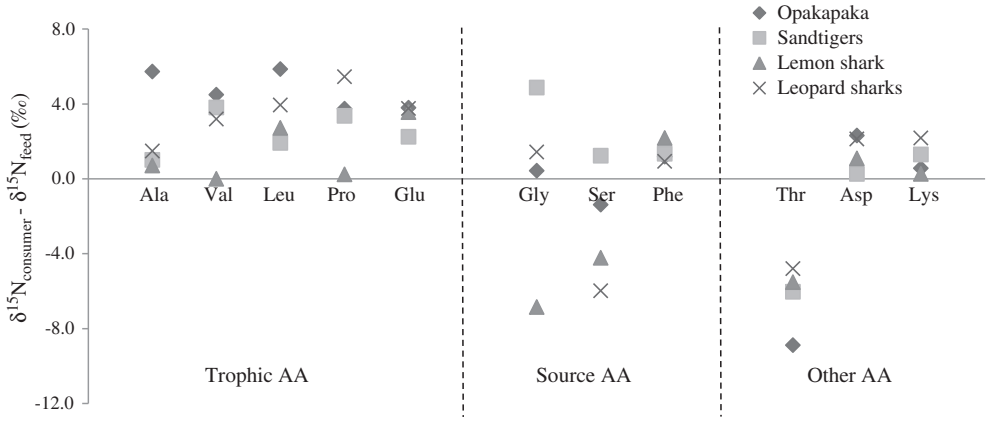

Fig. 2. Amino acid nitrogen isotopic enrichment between consumers and feed. Enrichment for both glycine and serine could not be determined for TD Sand tiger and DSW Sand tiger. Enrichment for alanine could not be determined for FS Leopard shark. Enrichment for lysine could not be determined for Opaka 5.

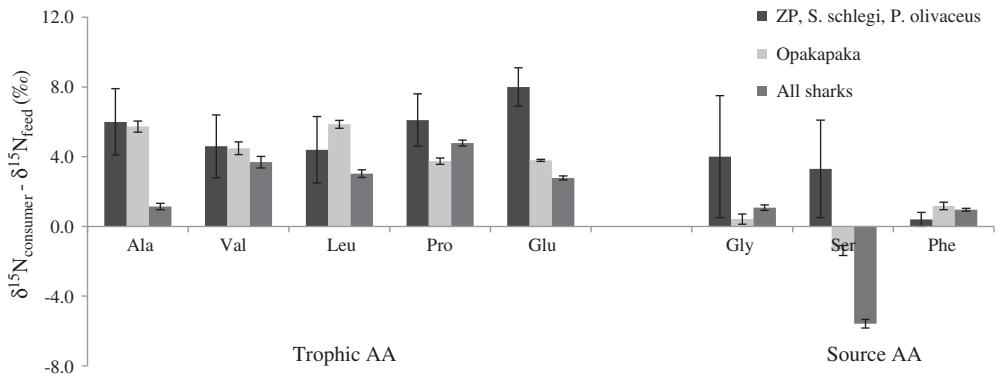

Fig. 3. Amino acid nitrogen isotopic enrichment between consumer and feed for opakapaka, sharks, and zooplankton (ZP), *S. schlegi*, and *P. olivaceus* reported by Chikaraishi et al. (2009). Error bars represent one standard deviation.

carnivorous diet could drive the low TEF values in these consumers. Previous studies have suggested that decreasing dietary protein quality or quantity increases ^{15}N trophic discrimination due to increased amino acid scavenging (Florin et al., 2011; Robbins et al., 2005, 2010; Vanderklift and Ponsard, 2003). The low TEF values found in this study of large, carnivorous fishes fed a high protein diet support this hypothesis but we have no data on these organisms fed a low-protein diet for comparison. It follows that higher TEF values would be expected in the trophic relationships of zooplankton and juvenile zooplanktivorous fish, *S. schlegi* and *P. olivaceus*, as these organisms were fed lower protein diets (Chikaraishi et al., 2009). While we cannot identify the exact mechanism of ^{15}N enrichment from our results, a lower TEF requires that less ^{14}N is lost from the organism. As phenylalanine Δ appears relatively constant, smaller TEF values with increasing TP imply decreasing deamination of glutamic acid and loss of ^{14}N as waste (urea or ammonium).

4.2. Potential urea effects

Despite similar $TEF_{glu-phe}$ values among sharks and opakapaka, we cannot entirely dismiss the hypothesis that urea production and retention lower shark TEF values. Elasmobranchs utilize glutamine as a nitrogen donating substrate for ammonia, while other taxa use alanine and aspartate (Anderson, 2001). Dale et al. (2011) suggested that this alternative pathway of urea synthesis could result in lower glutamic acid enrichment, however without knowledge of urea concentrations we are unable to comment on potential effects of urea on TEF values.

In addition to urea, elasmobranchs have trimethylamine N-oxide (TMAO) in their tissues to prevent protein destabilization due to urea. Concentrations of urea and TMAO are regulated concurrently according to ambient salinity and habitat depth, further complicating the urea effect on nitrogen isotope fractionation between diet and elasmobranch tissues (Hammerschlag, 2006; Laxson et al., 2011; Wood et al., 2007). The effect of TMAO on elasmobranch isotope and TEF values is not understood, as it is uncertain whether elasmobranchs biosynthesize TMAO or acquire it through diet (Ballantyne, 1997). If TMAO is acquired through diet, it is unlikely to affect $\delta^{15}N$ values of individual amino acids. If biosynthesis of TMAO affects the biosynthesis of amino acids, it may alter the $\delta^{15}N$ values of individual amino acids. Obtaining accurate $\delta^{15}N$ values for TMAO is an important first step in understanding these effects and should be assessed in future studies.

Table 4
TEF values and statistical results of Kruskal–Wallis test of TEF values between sharks, opakapaka, and zooplankton (ZP), and zooplanktivorous fish, *S. schlegi*, and *P. olivaceus* reported by Chikaraishi et al. (2009). Phenylalanine was used as the source AA in TEF calculations. VLP: valine–leucine–proline. AVLP: alanine–valine–leucine–proline.

Trophic AA	TEF (S.D.) (‰)			H	d.f.	p-Value
	ZP, *S. schlegi*, *P. olivaceus*	Opakapaka	All sharks			
Alanine	5.6 (1.9)	4.6 (0.4)	−0.1 (0.3)	13.28	2	0.0013*
Valine	4.2 (1.8)	3.0 (0.5)	1.8 (0.4)	5.35	2	0.0690
Leucine	4.0 (1.9)	4.8 (0.4)	1.8 (0.3)	11.42	2	0.0033*
Proline	5.7 (1.6)	2.7 (0.3)	2.5 (0.3)	11.89	2	0.0026*
Glutamic acid	7.6 (1.2)	1.7 (0.2)	2.3 (0.2)	17.31	2	0.0002*
VLP	4.8 (1.1)	3.0 (0.3)	2.3 (0.2)	10.36	2	0.0056*
AVLP	5.0 (1.0)	3.6 (0.3)	1.4 (0.2)	13.6	2	0.0011*

* Significant effects.

4.3. Other possible sources of variability

Although phenylalanine Δ values for sharks and opakapaka were slightly larger than the value found for zooplankton and juvenile zooplanktivorous fish (Chikaraishi et al., 2009), there is strong evidence that these consumers were in steady state with the isotopic composition of their diet. Leopard shark diet was carefully controlled and these consumers were found to be in isotopic steady state with their diet based on bulk isotopic results (Kim et al., 2012). Phenylalanine Δ values were more variable in sand tigers and opakapaka than leopard sharks, but once averaged there was good agreement in phenylalanine Δ between leopard sharks and the other consumer groups. Further, source AA Δ values were not likely biased by temporal variations in diet $\delta^{15}N$ values. Considering the lengths of time these consumers were in captivity, their maturity, slow growth, and low tissue incorporation rates, temporal variability in diet $\delta^{15}N$ values would be muted.

Differences in feed assimilation between consumers may be another source of intra-species TEF value and bulk tissue discrimination variability. For example, it was assumed that all opakapaka ingested the same amount of each food type (Table 3). If one fish had eaten more krill than others, it would have a different average $\delta^{15}N_{feed}$ value, altering TEF values and bulk discrimination factors. Further, we do not know if these consumers utilized dietary nitrogen from each feed source proportionately. However, it is unlikely that TEF values were significantly skewed by these factors. Leopard sharks were fed a single source diet and TEF values of these organisms were in close agreement with those of the other carnivorous consumers.

4.4. Application in food web studies

A critical question concerns the applicability of these results to wild populations. Consumers were kept in aquaria (sand tigers and lemon shark), tanks (leopard sharks), and cages (opakapaka) and fed consistently, limiting mobility and metabolic activity. Unsurprisingly, opakapaka muscle fat content was higher than that of wild-caught fish. Further, the organisms fed to these captive animals may not be representative of food that they might normally consume in the wild. For example, leopard sharks (fed cephalopods in this study) primarily feed on benthic invertebrates and fishes common in estuaries (Carlisle

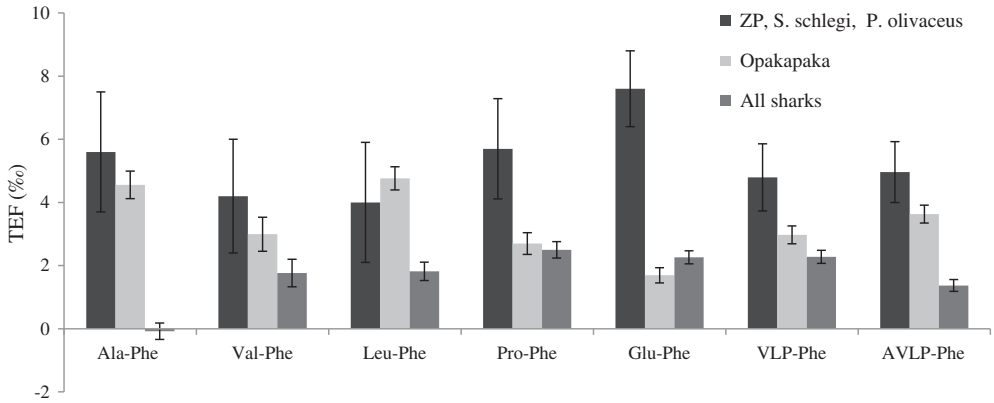

Fig. 4. TEF values for various trophic-source AA combinations for opakapaka, sharks, and zooplankton (ZP), *S. schlegi*, and *P. olivaceus* reported by Chikaraishi et al. (2009). Error bars represent one standard deviation. VLP and AVLP represent TEF values of weighted means for valine–leucine–proline and alanine–valine–leucine–proline, respectively.

and Starr, 2009). A constant food supply with high protein quality may have lowered isotopic fractionation. If this is the case, TEF values calculated from these captive reared fishes are lower than TEF values of wild opakapaka and shark populations.

Despite these uncertainties, our results clearly indicate that TEF values are variable among species for all analyzed trophic and source amino acids. It is possible there is a fractionation difference between grazers and omnivores/carnivores (due to differences in dietary protein quantity or quality), where 7.6‰ represents the TEF between autotroph and grazer, but the TEF of carnivores is <7.6‰. If true, TP calculations for wild fish populations should utilize an integrated TEF, which includes both values. For example, assuming that a TEF value of 7.6‰ is appropriate for herbivores, we suggest a potential equation of the form:

$$TP = \frac{\left(\delta^{15}N_{glu} - \delta^{15}N_{phe}\right) - \beta - TEF_{herbivore}}{TEF_{carnivore}} + 2 \quad (4)$$

where $\beta = 3.4‰$, $TEF_{herbivore} = 7.6‰$ and $TEF_{carnivore} < 7.6‰$ (Fig. 1B). This approach should be tested in natural food webs using marine herbivores, omnivores, and carnivores where the trophic ecology can be constrained.

5. Conclusions

This study found a considerable departure from the predominantly used $TEF_{glu-phe}$ of 7.6‰ in three elasmobranch species, sand tiger (*C. taurus*), lemon sharks (*N. brevirostris*), and leopard sharks (*T. semifasciata*) as well as one carnivorous teleost species, opakapaka (*P. filamentosus*), fed high protein diets. $TEF_{glu-phe}$ values for sharks and opakapaka were much lower than $TEF_{glu-phe}$ values previously reported from captive feeding experiments on zooplankton and juvenile zooplanktivorous fish (Chikaraishi et al., 2009). Similar $TEF_{glu-phe}$ values for sharks and opakapaka suggest differences in isotopic fractionation potentially resulting from dietary protein quantity or quality and do not support the hypothesis that TEF values are low in sharks due to urea retention. The applicability of these values to food web studies of wild teleost and shark populations remains uncertain, and the TEF values found through these captive feeding studies are likely an underestimation of TEF values for wild populations due to overfeeding and/or limited mobility. Nevertheless, these results clearly show variable and low TEF values particularly for mature carnivorous fish.

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