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Nitrogen isotope fractionation in amino acids from harbor seals: implications for compound-specific trophic position calculations

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ABSTRACT: Compound-specific isotope analysis of individual amino acids (AA) is a rapidly growing tool in ecological studies to assess diet and trophic position (TP) in both modern and ancient foodwebs. We conducted the first controlled feeding study examining $\delta^{15}N$ values in AAs in a marine mammal (harbor seal *Phoca vitulina*). The pattern of $\delta^{15}N$ variation among AAs in seals was similar to that observed in other heterotrophs, although exceptions were found with proline and threonine. However, many $\delta^{15}N$ changes with trophic transfer were very different than those reported for zooplankton and other lower TP marine consumers. In particular the measured trophic enrichment factor (TEF) now broadly used for TP estimation ($TEF_{Glu\text{-}Phe}$) was much lower in harbor seals (~4.3‰) than the current commonly applied value (~7.5‰). Recently published data on wild marine birds (penguins) and elasmobranchs (stingrays) suggests that similar, low TEF values may also be characteristic of these taxa. Together, these data imply that marine mammals and other higher animals have different, but also diagnostic, changes in $\delta^{15}N$-AA with trophic transfer vs. organisms examined in previous feeding studies (e.g. zooplankton, bony fish and mollusks), possibly due to dietary protein content, trophic position, and/or form of nitrogen excretion (urea vs. ammonia). Therefore, we propose that for marine mammals, a multi-TEF calculation is required to account for variations of TEF between animals within a food web, and we demonstrate that this approach can predict accurate TP estimates for harbor seals. These results also have significant implication for the application of compound-specific isotope analysis of AAs on terrestrial ecology and trophic structure.

KEY WORDS: Compound-specific isotope analysis · Trophic enrichment factors · *Phoca vitulina* · Trophic position · TEF · Urea · Ammonia · Protein content

INTRODUCTION

Stable isotope analysis has been widely used to understand the diet, migration, and food web dynamics of marine animals, greatly increasing understanding of complex ecosystems and elusive organisms, and broadening the scope of research on both living and fossil marine mammals (e.g. Koch 2007, Newsome et al. 2010). Most such studies have used carbon and nitrogen isotope ($\delta^{13}C$ and $\delta^{15}N$) analysis of bulk tissues (e.g. muscle, feather and red blood cells; Kelly 2000). However, bulk isotopic approaches have inherent limitations, such as the potential for unrecognized variation in isotopic values at the base of food webs, uncertainties about isotopic routing of macromolecules during tissue synthesis, and variable trophic enrichment factors (Martínez del Rio et al. 2009, Newsome et al. 2010). In the last 2 decades, a new approach, compound-specific isotope analysis, has grown rapidly in isotope ecology, providing more

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information about an organism's physiology and biochemistry (Hare et al. 1991, Fogel et al. 1997, McClelland & Montoya 2002, Fogel & Tuross 2003, Corr et al. 2005, Popp et al. 2007, Martínez del Rio et al. 2009). Isotopic analysis of individual organic compounds now has the potential to become relatively routine with the advent of coupled GC and isotope-ratio-monitoring mass spectrometry (GC-IRMS).

Compound-specific isotope analysis of individual amino acids (AA) is a particularly powerful approach because it has the ability to greatly refine information about the dietary behavior, trophic dynamic interactions, microbial degradation, and nutritional status of plants and animals (e.g. McCarthy et al. 2004, 2007, Popp et al. 2007, Martínez del Rio et al. 2009, McMahon et al. 2010, Larsen et al. 2011). Early work revealed that specific fractionation patterns among AAs in ^{15}N-to-^{14}N for autotrophic organisms are related to specific biochemical pathways (Abelson & Hoering 1961, Macko et al. 1987), and the pattern of isotopic values in a heterotroph thus reflects both the autotrophic biochemical signature and subsequent trophic transfers (McCarthy et al. 2004, 2007). The $\delta^{15}N$ measurements of individual AAs ($\delta^{15}N$-AA) have generated the most intense recent interest, because of their potential to decouple variation in $\delta^{15}N$ values at the base of food webs from isotopic effects of trophic transfer (e.g. Popp et al. 2007, Sherwood et al. 2011), simultaneously offering a new tool for far more precise estimates of trophic position (TP) in animals (e.g. Chikaraishi et al. 2009).

Trophic position estimates based on $\delta^{15}N$-AA values are based on variable $\delta^{15}N$ enrichment of individual AAs with trophic transfer. McClelland & Montoya (2002) were the first to show a sharp distinction in ^{15}N-enrichment between different AAs with trophic transfer: approximately half of the commonly measured protein AAs become strongly ^{15}N-enriched with each trophic step, whereas another group of AAs largely maintain their $\delta^{15}N$ values from the base of the food web. These groups are now commonly called 'trophic' (i.e. those that enrich with trophic transfer) and 'source' (i.e. those that do not enrich with trophic transfer), respectively (after Popp et al. 2007). While conceptually similar to essential vs. non-essential AA groupings (based on the ability of organisms to synthesize R-groups), there is little overlap between these classifications. Of particular interest, several source AAs (e.g. lysine, phenylalanine, tyrosine, methionine) are believed to undergo little or no transamination in consumers, so that they provide a direct proxy for $\delta^{15}N$ values at the base of food webs (McClelland & Montoya 2002, Chikaraishi et al. 2009). In contrast, the trophic AAs are central to cycling nitrogen in and out of the AA pool (e.g. alanine, glutamate and aspartate), so they are strongly ^{15}N-enriched relative to diet. This observation that source and trophic AAs show differential ^{15}N-enrichment relative to the same AA in diet ($\Delta = \delta^{15}N_{AA\ in\ consumer} - \delta^{15}N_{AA\ in\ food}$) forms the basis for using compound-specific isotope analysis to estimate TP in food webs (McClelland & Montoya 2002, Chikaraishi et al. 2009).

A critical underlying issue for using this approach to interpret trophic structure of wild populations is therefore the magnitude of ^{15}N-enrichment with each trophic step. Previous research has suggested that ^{15}N-enrichment in trophic AAs with each trophic step be normalized to the much smaller ^{15}N-enrichment in source AAs in the form of a 'trophic enrichment factor' (TEF; McClelland & Montoya 2002, Chikaraishi et al. 2009). To date, studies have focused primarily on the TEF for glutamic acid (Glu) and phenylalanine (Phe), representing the most consistent of the trophic and source AA groups respectively ($TEF = \Delta_{Glu} - \Delta_{Phe}$; e.g. McClelland & Montoya 2002, Chikaraishi et al. 2009). If TEF remains constant for all steps in a food chain, then the TP of a consumer can be estimated from the difference in $\delta^{15}N$-AA values between source and trophic AAs (e.g. Chikaraishi et al. 2009). This estimate will then be independent of both food sources and $\delta^{15}N$ values at the base of the food web, making TEF values the key variable underlying the accuracy of $\delta^{15}N$-AA based TP estimates (McClelland & Montoya 2002, McCarthy et al. 2007, Popp et al. 2007, Dale et al. 2011).

Prior feeding studies, performed exclusively on plankton and lower TP marine organisms, have yielded fairly constant TEF values (McClelland & Montoya 2002, Chikaraishi et al. 2007, 2009). Specifically, Phe has been found to undergo almost no change with trophic transfer ($\Delta_{Phe} \approx 0$ to 0.4 ‰), whereas Glu typically shows greatest enrichment ($\Delta_{Glu} \approx 7.0$ to 8.0‰), leading to a $TEF_{Glu\text{-}Phe}$ value of ~7.6‰ (McClelland & Montoya 2002, Chikaraishi et al. 2009). These values have now been widely applied to calculate TP in diverse applications, including planktonic ecosystems (McClelland et al. 2003, Schmidt et al. 2004, McCarthy et al. 2007, Hannides et al. 2009), wild top consumers (e.g. tuna, penguins; Popp et al. 2007, Lorrain et al. 2009), and fossil human skeletal remains (Naito et al. 2010, Styring et al. 2010). Calculated TP results from field studies have mostly been consistent with the assumption of similar TEF values. However, recent data from some wild top marine carnivores also strongly suggest that

the core assumption of constant TEF values may need to be reevaluated for some animal groups (Lorrain et al. 2009, Dale et al. 2011), especially since an intermediate-top predator, such as a harbor seal, breaks down nitrogen differently to lower trophic organisms. Thus, TEF values and their corresponding trophic information may need to be reconsidered in future top consumer studies.

Despite the enormous potential of δ^{15}N-AA analysis for ecological studies of carnivores that are elusive or difficult to observe (such as marine mammals), there are currently no controlled experimental feeding studies that have examined how δ^{15}N values in AAs change with trophic transfer in any carnivorous vertebrate. We address this issue through compound-specific isotope analysis of samples from a feeding study on harbor seals *Phoca vitulina richardii*, a small pinniped found in coastal waters from Baja California to the Aleutian Islands (Carretta et al. 2001). Our main goal was to test the assumptions underlying the current application of compound-specific amino acid TP studies for a marine mammal; specifically, to determine whether the overall δ^{15}N-AA patterns from a common marine mammal are comparable to those determined previously for lower TP organisms, and to examine whether the now widely used TEF values and equations originally derived from plankton are applicable for marine mammal studies.

MATERIALS AND METHODS

Harbor seal and herring sampling

Harbor seal serum and muscle tissue were collected in spring 2007 from the Marine Mammal Center (TMMC), a rescue and rehabilitation center in Sausilito, California. All serum samples were collected from individual weaner seals (<1 yr old, n = 7; see Table 1), directly before release to the wild or medically-required euthanasia. Time at TMMC ranged from 1 to 93 d, and only serum from seals fully equilibrated to diet and considered nutritionally healthy (>14 d; as previously demonstrated using bulk isotope data; Germain et al. 2012) were used for TEF and TP calculations. Isotopic equilibrium specifically for AA was also directly tested using compound-specific δ^{13}C measurements (Fig. 1, see discussion below). Blood samples were collected in no-additive collection tubes from the epidural vein and allowed to separate for an hour. The blood was centrifuged, and the serum was collected and stored at −80°C. Pectoral muscle tissue was also collected from post-mortem individuals (n = 8) and stored at −80°C (see Table S1 in the Supplement at www.int-res.com/articles/suppl/m482p265_supp.pdf). Pectoral muscle samples were used here only to compare TEF and TP values between tissue types (Table S1), but were not combined with serum data in order to derive TEF or TP calculations.

Seals at TMMC were fed Atlantic herring *Clupea harengus* exclusively, which were wild caught in the same local fishery near Nova Scotia, and stored frozen in a large batch to provide food for all rehabilitating pinnipeds. The very young animals were fed a ground mixture for the first few weeks until their teeth emerged and they were able to swallow whole fish. We therefore expect that all herring batches would have similar δ^{15}N-AA values, as they were sourced from similar ocean regions and time frames. Nevertheless, to account for possible variation in δ^{15}N values from different herring batches, fish samples were collected from multiple batches throughout the experimental period (~15 fish each from 3 batches), combined, and ground into a single homogenous mixture for our compound-specific isotope analysis. Analysis of this herring homogenate represents the 'fish feed' endmember of the feeding study analysis.

To further verify the diet-to-tissue equilibrium, specifically for the AA pool between the composite herring and analyzed harbor seal serum, as noted

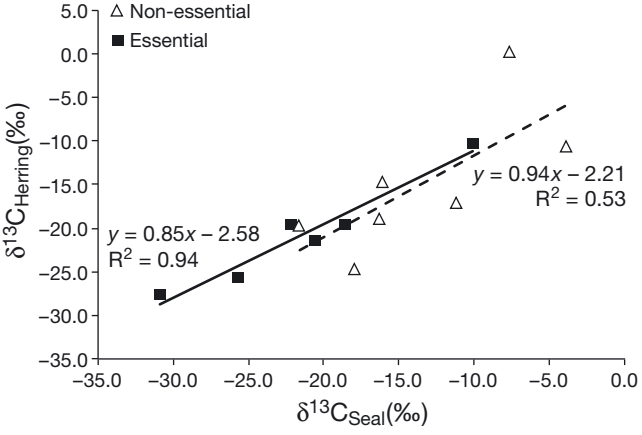

Fig. 1. Linear regressions of $\delta^{13}C_{Seal}$ vs. $\delta^{13}C_{Herring}$ for essential and non-essential AA groupings. Carbon in essential AA (squares) can be derived only from food sources, and therefore a high R^2 value indicates complete equilibration (at least for the AA pool) between animal and diet (e.g. O'Brien et al. 2005, McMahon et al. 2010). In contrast, carbon skeletons of non-essential AA (triangles) are resynthesized to varying degrees in the animal consumer, and are therefore expected to have a much lower R^2 value

above $\delta^{13}C$-AA values were also measured in both seal serum and the homogenized herring samples. The $\delta^{13}C$ values of essential AAs have carbon skeletons that animals cannot synthesize (i.e. they are derived directly from diet). It has been shown that $\delta^{13}C$-AA values for essential AAs are unchanged with trophic transfer (in contrast to non-essential AAs which are resynthesized to varying degrees), and therefore correlate strongly with the $\delta^{13}C$ value of the same AA group in diet, when diets are fully equilibrated (O'Brien et al. 2005, McMahon et al. 2010). Linear regressions of $\delta^{13}C$ values of the essential AAs (Fig. 1) for seals vs. the herring food mixture shows a high correlation ($R^2 = 0.94$), confirming the expected diet equilibration and direct trophic linkage between the AAs in our sampled seals and the same AA composite herring food sample. This result is consistent with previous bulk $\delta^{15}N$ data (Germain et al. 2012), which reached equilibrium as well. A full discussion of $\delta^{13}C$ values and methods is beyond the scope of this paper; however, additional methodology and details are supplied in the supplement (available at www.int-res.com/articles/suppl/m482p265_supp.pdf).

Bulk and compound-specific amino acid isotope analyses

Measurement of bulk $\delta^{13}C$ and $\delta^{15}N$ values have been described previously for these samples (Germain et al. 2012). Briefly, serum, muscle and herring were lyophilized, homogenized, and desiccated following the protocols of Dobush et al. (1985). Total lipid extraction was performed on dried samples using a Dionex Accelerated Solvent Extractor, where the samples were rinsed twice with 100% petroleum ether at 50°C and 1500 psi (~10 MPa), held in 60% volume for 5 min, and finally dried under a fume hood to remove residual solvent. Isotopic results are expressed in parts per thousand (ml^{-1}; ‰) as: $\delta^{13}C$ or $\delta^{15}N = ([R_{sample}/R_{standard}] - 1) \times 10^3$, where R_{sample} and $R_{standard}$ are the $^{13}C/^{12}C$ or $^{15}N/^{14}N$ ratios of the sample and standard, respectively. The standards are Vienna-Pee Dee Belemnite (V-PDB) for carbon, and atmospheric N_2 for nitrogen.

The $\delta^{15}N$ values of individual AAs were measured via GC-IRMS, after 6 N HCl acid hydrolysis and the formation of TFA ester derivatives, following published McCarthy lab protocols (e.g. McCarthy et al. 2007, Sherwood et al. 2011); more detailed compound-specific isotope AA analytical descriptions are also provided in the Supplement. We determined $\delta^{15}N$ values for 14 AAs, each measured 4 times: alanine (Ala), aspartic acid (Asp), glutamic acid (Glu), isoleucine (Ile), leucine (Leu), proline (Pro), valine (Val), glycine (Gly), lysine (Lys), serine (Ser), threonine (Thr), tyrosine (Tyr), phenylalanine (Phe), and arginine (Arg). Due to cleavage of the terminal amine groups in glutamine (Gln) and aspartamine (Asn), Gln is converted to glutamic acid (Glu), and aspartamine (Asn) is converted to aspartic acid (Asp) during acid hydrolysis. This results in the measurement of combined Gln + Glu (referred to hereby as Glu), and Asn +Asp (referred to hereby as Asp). We are aware some researchers refer to these groupings as Glx and Asx; however, we chose our terminology here to be consistent with other compound-specific isotope studies. These were categorized into 3 groupings: source AAs (Gly, Lys, Ser, Tyr, Phe, and Arg), trophic AAs (Ala, Asp, Glu, Ile, Leu, Pro, and Val), and metabolic (Thr).

TEF, TP estimates, and statistical tests

We define the TEF for Glu and Phe, which are generally accepted as the best trophic and source AAs for TP estimates, as follows:

$$TEF_{Glu\text{-}Phe} = \Delta_{Glu} - \Delta_{Phe} = (\delta^{15}N_{Glu,Consumer} - \delta^{15}N_{Glu,Food}) - (\delta^{15}N_{Phe,Consumer} - \delta^{15}N_{Phe,Food}) \quad (1)$$

Alternate TP estimates based only on the bulk $\delta^{15}N$ values were also determined, as described in Germain et al. (2012):

$$TP_{Bulk} = [(\delta^{15}N_{Serum} - 3.8) - \delta^{15}N_{Plankton})/3.4] + 2 \quad (2)$$

where 3.8 represents one bulk trophic transfer between a harbor seal and herring; 3.4 is one trophic transfer between herring and plankton; and +2 is to account for those 2 trophic steps.

Seal TP estimations were determined using compound-specific isotope analysis of AA differences between Glu and Phe, using either the currently widely used single-TEF approach (after McClelland & Montoya 2002) or the new multi-TEF equation proposed here. For the single TEF approach, we used the equation of Chikaraishi et al. (2009):

$$TP = [(\Delta^{15}N_{Glu\text{-}Phe,Seal} - 3.4) / TEF_{Glu\text{-}Phe}] + 1 \quad (3)$$

where $\Delta^{15}N_{Glu\text{-}Phe,Seal} = \delta^{15}N_{Glu} - \delta^{15}N_{Phe}$ measured in the seal serum or muscle; 3.4 represents $\delta^{15}N_{Glu} - \delta^{15}N_{Phe}$ in primary producers; and $TEF_{Glu\text{-}Phe} = 7.6$‰ average value for plankton.

The multiple-TEF equation (as described in the 'Results and discussion' section and also derived in the Supplement), accounts for both calculated TEF's

Table 1. *Phoca vitulina*. Harbor seal amino acid $\delta^{15}N$. All harbor seals were <1 yr old. All AA measurements were replicated 4 times (± SD reported for each AA in parentheses) with an average SD of 0.8‰. Fraction: Serum (S), Whole (W); Sex: Male (M), Female (F); Days: number of days at TMMC when tissue sampling occurred. Health status scale of 1–7 (where 1 = starving, 3 = healthy, and 7 = obese) indicates nutritional state according to blubber thickness. Data and TEF calculations within the text are extrapolated from serum samples for seals in TMMC longer than 2 wk (i.e. equilibrated diet) compared to ground-up fish feed

Sample no.	Fraction	Sex	Days	Health	Status	Ala	Gly	Thr	Ser	Val	Leu	Ile	Pro	Asp	Glu	Phe	Lys	Tyr	Arg	Bulk
1696	S	M	62	–	Released	27.2 (0.8)	12.9 (1.6)	–17.0 (0.6)	9.1 (2.1)	23.5 (2.0)	24.0 (0.8)	15.5 (2.0)	28.1 (0.6)	18.1 (0.2)	23.7 (0.7)	9.5 (0.8)	14.0 (0.6)	20.3 (0.8)	17.7 (0.6)	16.4
1698	S	F	83	–	Released	24.1 (0.3)	10.3 (0.7)	–25.4 (0.6)	9.7 (0.7)	17.7 (0.3)	23.7 (0.5)	19.4 (0.3)	29.4 (0.4)	18.7 (0.4)	23.4 (0.4)	10.2 (0.4)	13.5 (0.4)	19.0 (0.6)	18.6 (0.2)	16.5
1704	S	F	36	3	Colitis (died)	22.1 (0.5)	12.7 (0.7)	–22.8 (1.2)	10.8 (0.6)	19.7 (0.5)	23.2 (0.2)	22.7 (0.5)	28.7 (0.6)	17.5 (0.4)	22.7 (0.1)	10.7 (0.6)	5.6 (1.4)	19.3 (0.5)	–	15.8
1705	S	F	93	–	Released	23.3 (0.8)	9.6 (0.5)	–27.5 (0.9)	9.1 (0.4)	17.1 (0.8)	23.3 (0.2)	21.5 (1.1)	28.3 (0.5)	18.2 (0.3)	23.3 (0.6)	9.6 (0.8)	11.5 (1.2)	17.3 (0.3)	15.8 (3.7)	16.3
1718	S	F	62	6	Neurological (euthanized)	21.9 (0.4)	10.2 (0.6)	–18.6 (1.3)	9.6 (0.7)	22.1 (0.5)	22.8 (0.7)	18.2 (0.9)	26.8 (0.5)	18.8 (0.4)	24.4 (0.4)	9.1 (1.0)	7.0 (1.4)	19.6 (0.7)	–	15.4
1739	S	F	18	3	Aspiration; enteritis (euthanized)	20.1 (0.8)	15.2 (2.3)	–7.6 (1.2)	13.0 (1.2)	16.7 (1.2)	17.1 (0.5)	1.8 (0.7)	21.4 (0.4)	16.2 (0.8)	19.7 (0.5)	11.9 (0.7)	3.5 (1.0)	20.7 (0.8)	9.2 (1.2)	12.4
1748	S	M	1	3	Spine trauma (euthanized)	26.8 (1.5)	18.9 (1.0)	–21.4 (0.6)	19.8 (1.4)	26.6 (1.3)	25.8 (0.5)	20.8 (2.1)	29.0 (0.5)	21.4 (0.5)	25.8 (0.6)	12.7 (0.9)	16.7 (0.9)	14.1 (0.8)	–	18.0
Fish Feed	W					21.9 (0.6)	4.3 (0.4)	–13.6 (1.7)	1.7 (0.6)	13.8 (1.7)	21.7 (0.6)	21.2 (0.7)	22.7 (0.6)	15.7 (1.2)	20.6 (0.7)	11.3 (4.4)	8.6 (1.0)	14.1 (0.7)	–	12.5

in multiple organisms commonly found in a marine food web:

$$TP = [(\Delta^{15}N_{Glu\text{-}Phe.Seal} - TEF_{Seal}) / TEF_{Plankton}]) + 2 \quad (4)$$

where TEF_{Seal} is the $TEF_{Glu\text{-}Phe}$ value for seals (and possibly other mammals), and $TEF_{Plankton}$ is the $TEF_{Glu\text{-}Phe}$ value previously found in plankton and also other lower marine organisms (Chikaraishi et al. 2009).

The differences for both $TEF_{Glu\text{-}Phe}$ and Δ values for individual AAs between our data and those reported in Chikaraishi et al. (2009) were compared using *t*-tests and one-way analysis of variance (ANOVA). The average analytical standard deviation for both Glu and Phe are 0.5‰ and 0.7‰, respectively. We note that for CSI-AA, in general, approximately ±1‰ is a fairly typical precision achieved, so in fact this level of variation is very good to excellent. With regard to the basic level of significance, we have propagated these errors using standard error propagation formulas. Propagated errors for TEF ranged from 0.6‰ to 1.1‰, resulting in the highest TEF value of 7.0‰ and all other TEF's below 5.9‰ (significantly lower than found in Chikaraishi et al. 2009). Significant results are reported when p-values were less than 0.05. Statistical analyses were performed in JMP (version 7).

RESULTS AND DISCUSSION

Overall $\delta^{15}N$-AA patterns and values

We present the first patterns for $\delta^{15}N$-AA values reported for a living pinniped (Table 1, Fig. 2). The $\delta^{15}N$ values for 7 harbor seals were grouped together as either 'trophic' or 'source' AAs following the order proposed in McClelland & Montoya (2002), as well as a new 'metabolic' (MB) designation for Thr. The pattern in $\delta^{15}N$-AA values for all seals generally corresponds well with expectations from prior studies. The source AAs all have lower $\delta^{15}N$ values, presumably reflecting more closely the $\delta^{15}N$ values of AAs in autotrophs at the base of the food web (Popp et al. 2007), whereas the trophic AAs all have significantly elevated $\delta^{15}N$ values, linked to ^{15}N enrichment from deamination/transamination associated with trophic transfer (Macko et al. 1987, McClelland & Montoya 2002). The $\delta^{15}N$ value of Phe in particular, because it is linked directly to $\delta^{15}N$ values at the base of the food web, has been used to assess the foraging behavior of animals over wide marine spatial scales. For example, Phe $\delta^{15}N$ values have been observed in yellowfin tuna ranging from 3–10‰, in Southern Ocean penguins from 1–5‰, in coastal stingrays from 2–6‰, and in these harbor seals from 9–12‰ (Popp et al.

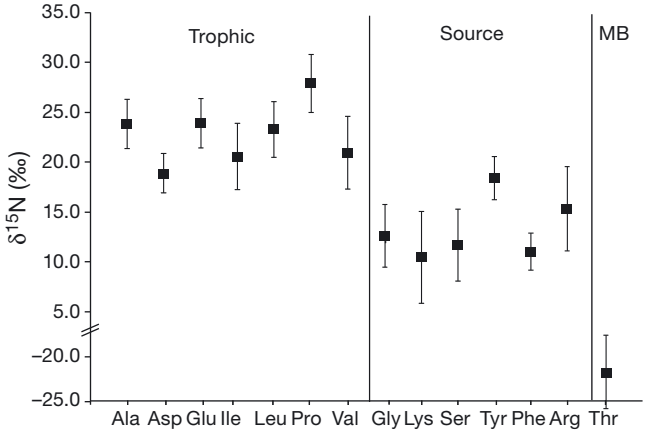

Fig. 2. *Phoca vitulina*. Harbor seal δ^{15}N-AA pattern. Measured (i.e. non-normalized) individual amino acid data are arranged according to the trophic, source and metabolic (MB) amino acid groupings. Error bars = 1 SD (n = 7 seals); AA abbreviations are defined in 'Materials and methods'. Note scale break required for inclusion of extremely depleted Thr δ^{15}N value

2007, Lorrain et al. 2009, Dale et al. 2011). In each case, the range in these isotopic values is typically interpreted as indicating feeding area, across ocean regions with shifting δ^{15}N isotopic baselines. Thus, this isotopic tool can be useful in predicting the foraging movement and location of marine predators over different time periods. The complete AA pattern in seals can also be compared more widely with previous data for diverse heterotrophic organisms (see Fig. S1 in the Supplement); all data sets have similar patterns that hold across diverse groups of organisms, and all are consistent with the expectation of ^{15}N-enrichment for trophic AAs and relative δ^{15}N stability for source AAs. This agreement underscores that basic δ^{15}N-AA patterns, as well as relative behavior, of trophic and source AA categories seem universal.

However, in harbor seals there are also several AAs that depart from the δ^{15}N patterns observed in most previous work. One notable difference is that in each of the harbor seals sampled in this study, Pro was consistently the most ^{15}N-enriched AA. In prior studies on plankton and fish, Glu is typically observed as the most enriched AA (e.g. McClelland & Montoya 2002, Bloomfield et al. 2011) consistent with its biochemical role as the primary N source pool for transamination of all other AAs (e.g. Champe & Harvey 2010). However, several recent studies on higher TP animals have also reported Pro to be the most ^{15}N-enriched AA (e.g. penguins and ancient humans; Lorrain et al. 2009, Styring et al. 2010), and it has been proposed that Pro might potentially replace Glu in TP calculations (Chikaraishi et al. 2009). Since Glu is well established as the most ^{15}N-enriched AA in autotrophs (e.g. Macko et al.1987, McClelland & Montoya 2002, Chikaraishi et al. 2009), the elevated Pro δ^{15}N in our seals and recent literature data suggests that Pro has variable enrichment with trophic transfer (i.e. that $\Delta_{Pro} > \Delta_{Glu}$ in selected higher TP organisms). An additional possible factor in seals might be the central role of Pro in the formation of collagen. Collagen is important in skin, bone, and connective tissues (comprising ~20% of the body mass and nearly 30% of protein mass of humans; Di Lullo et al. 2002), and every third AA in collagen is either Pro or hydroxyl-Pro. Linkage of elevated Pro δ^{15}N values with collagen synthesis would also be consistent with the observation that both muscle and bone tend to show similar elevated Pro values vs. Glu (Fig. 2). Overall, while Pro δ^{15}N values might thus provide a unique tracer in some animals, the apparent variation in Δ values (also seen in comparisons of lower TP organisms; Chikaraishi et al. 2009) suggests it is a poor choice for determining TP.

Thr has notably divergent δ^{15}N values vs. other AAs (Fig. 2). The extreme Thr ^{15}N-depletion observed in seals is consistent with some past heterotroph data (Hare et al. 1991, Popp et al. 2007, Sherwood et al. 2011; Fig. S1 in the Supplement), but it contrasts sharply to Thr values observed in both zooplankton and phytoplankton (McClelland & Montoya 2002, McCarthy et al. 2007, Chikaraishi et al. 2009). Together, these data clearly indicate that Thr is not a 'source' AA, as designated in early work, but instead that Thr becomes progressively depleted not only with increasing TP, but also to a far greater extent in at least some long-lived organisms (Fig. S1). For these reasons, we have designated it a 'metabolic' AA in our figures (MB). Factors proposed for low Thr values include successive trophic transfers (Styring et al. 2010), and also nutritional stress (Hare et al. 1991)—both of which are potential factors in these seals. However, comparison with other data also suggests that Thr depletion may be especially great in marine consumers (harbor seals, a whale, fossil marine mammals and marine-prey consuming humans; Fig. S1). We note that Thr (like Pro) is also biochemically involved in the formation of collagen and elastin (Bowes & Kenten 1949), suggesting that lower values in marine mammals could also be linked in part with blubber formation. While our data cannot differentiate between specific mechanisms, they do indicate that Thr (like Pro) does not follow expected trophic enrichment patterns from experiments on zooplankton (McClelland & Montoya 2002, Chikaraishi et al. 2009).

Harbor seal δ^{15}N-AA change with trophic transfer: Δ and TEF values

Δ values from a recent compilation of trophic transfer data for δ^{15}N-AA values (Chikaraishi et al. 2009) are compared with those found in harbor seals in this study (Fig. 3). As noted above, the Δ values are the difference of an individual AA between consumer and food, with a single trophic transfer. We observed large differences in Δ values vs. those determined in lower TP marine organisms (Fig. 3). Because of variation in the exact AAs that can be measured by different analytical approaches, it is not possible to directly compare Δ values for all AAs; however, for the majority of AAs in harbor seals (Ala, Glu, Ile, Leu, and Ser), values differ significantly from those determined in lower TP marine organisms ($p < 0.05$), many by large magnitudes. Based on past work, the Δ values for the trophic AA group would be expected to be relatively similar (e.g. McClelland & Montoya 2002). Because we also have the most directly comparable AA data for trophic AAs, the differences in this group seem particularly clear: 5 of 7 trophic AAs have Δ values much lower than would be expected based on past work (Fig. 3). The overall average Δ value for the trophic AA group (2.7 ± 1.6‰) was also substantially lower than a corresponding average for the same AA reported previously (5.3 ± 1.8‰; Chikaraishi et al. 2009).

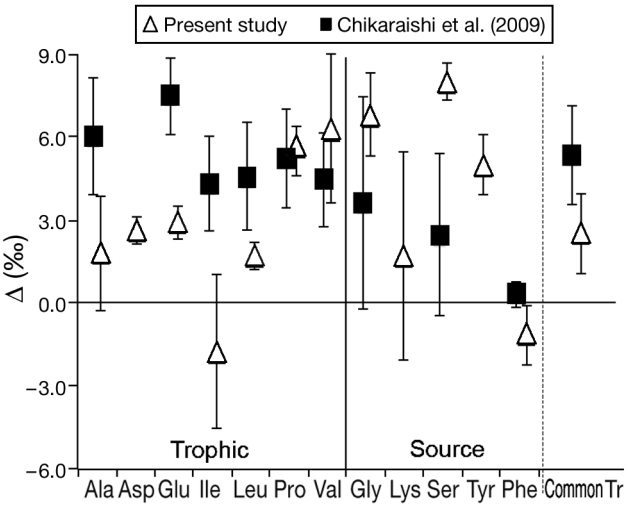

Fig. 3. Trophic transfer effect on individual δ^{15}N-AA values for harbor seals *Phoca vitulina*. Δ ($\delta^{15}NAA_{consumer} - \delta^{15}NAA_{food}$) values for harbor seals in the present study (open triangles; error bars = 1 SD, n = 6) contrasted against average $\Delta\delta^{15}N_{ConsumerAA\text{-}FoodAA}$ values determined for a range of lower TP marine consumers (black squares; all reported to have similar Δ values; Chikaraishi et al. 2009). Error bars for Chikaraishi data = 1 SD for the combined averages of zooplankton, gastropods, and fish (n = 4, 3, 2 respectively; Asp, Lys, Tyr not reported). Amino acid abbreviations are as defined in 'Materials and methods'. Common Tr = average of all trophic AA Δ values that were directly comparable between data sets

The Δ values for Phe, however, remain closest to 0‰ in both data sets (Fig. 3). While our average Phe value is near 1 standard deviation of 0‰, it also appears slightly depleted in seal consumers vs. herring food. While early work suggested Δ values for Phe were essentially 0‰, some variability in Δ values has also been observed. Chikaraishi et al. (2009) for example, suggest an average Phe Δ value of +0.4‰, although negative Δ values were also observed in some reported experiments. Field data from mixed zooplankton at specific sampling sites have also indicated consistently negative Phe Δ values (McClelland & Montoya 2002), similar in magnitude with the average Phe Δ value we observed here. Together, this suggests that more firmly determining possible ranges for Phe Δ values in diverse organisms may be important for precise trophic position calculations. Overall, however, our data is fully consistent with past work indicating that Phe is the AA which, on average, fractionates least from diet, further strengthening the argument that Phe is the 'best' (and perhaps only) functional source AA, and therefore represents the most accurate AA proxy for δ^{15}N at the base of food webs.

In contrast, the Δ value for Glu, which as noted above is typically used as the main 'trophic' AA for TP calculations, is one of the most strongly offset in harbor seals vs. previous work (Fig. 3). In prior feeding experiments with invertebrates, Glu has almost always shown the largest Δ value of any AA (average Δ = ~7.5‰; McClelland & Montoya 2002, Chikaraishi et al. 2009). The Δ value for Glu in harbor seals (Δ = 2.9 ± 0.6‰) is much lower than these previous values from feeding experiments, although it is quite similar to the Δ values for all the other main trophic AAs (Ala, Asp, and Leu). Together, these observations suggest that the nitrogen metabolism of many AAs with trophic transfer, and particularly those in the trophic group, may be quite different in harbor seals (and likely other mammals), vs. the invertebrates and fish used to determine Δ and $TEF_{Glu\text{-}Phe}$ values currently widely applied. The lower Glu Δ values, coupled with stable Phe δ^{15}N, result in a sharply lower $TEF_{Glu\text{-}Phe}$ value for harbor seals. Our data indicates a $TEF_{Glu\text{-}Phe}$ for harbor seals feeding on herring of 4.3 ± 1.2‰, about half of the typically assumed value (7.6 ± 0.4‰) derived from zooplankton, gastropods, and fish (McClelland & Montoya 2002, Chikaraishi et

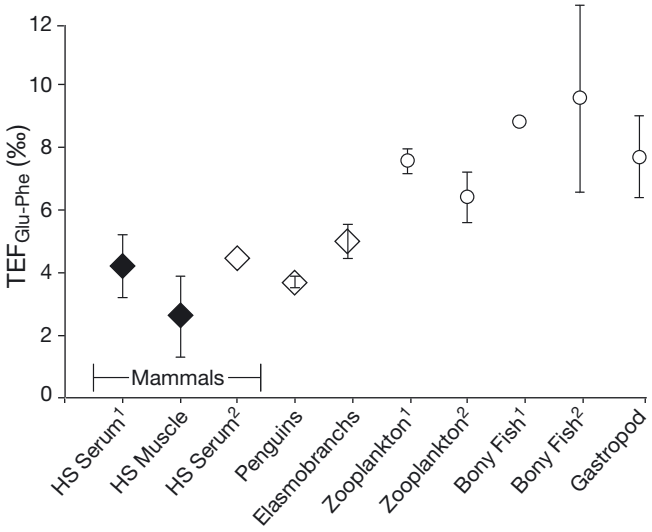

Fig. 4. Comparison of trophic enrichment factor ($TEF_{Glu\text{-}Phe}$) data for different animal groups. Bifurcation in existing $TEF_{Glu\text{-}Phe}$ data suggests that distinct ranges exist for organisms, possibly linked to major nitrogen waste product and/or elevated TP. Filled diamonds = data from this study (harbor seal serum[1] and muscle). Open diamonds = past literature data for harbor seals (harbor seal serum[2] is from Zhao 2002[1]), penguins (Lorrain et al. 2009), and elasmobranchs (stingrays and sharks; Dale et al. 2011). Open circles = ammonia-excreting organisms (zooplankton[1], bony fish[1], and gastropods from Chikaraishi et al. 2009; zooplankton[2] from McClelland & Montoya 2002; bony fish[2] from Bloomfield et al. 2011). Error bars = 1 SD

al. 2007). An unpublished PhD dissertation by Zhao[1] also measured compound-specific isotopes of AA in serum of one adult harbor seal and its food, using an alternate off-line analytical approach (Zhao 2002)[1]. While the analytical methods are quite different, the $TEF_{Glu\text{-}Phe}$ value derived from this data of 4.5‰ (Fig. 4) is also low, and in fact identical within error to our data for the same species. The essentially identical $TEF_{Glu\text{-}Phe}$ value derived from the only prior marine mammal feeding experiment conducted with an adult seal (Zhao 2002)[1], coupled with the similar low TEF values for several other species (fast growing penguins in Lorrain et al. 2009), suggests age/growth status as an unlikely explanation for the low TEF values across several different species shown in Fig. 4. Wild stingrays (muscle tissue samples) have also recently been estimated to have low $TEF_{Glu\text{-}Phe}$ values (5.0 ± 0.6‰; Dale et al. 2011), based on TP independently estimated using stomach content analysis, coupled with δ^{15}N-AA values. In contrast, data from some bony and larval fish species recently examined have suggested even higher $TEF_{Glu\text{-}Phe}$ than original zooplankton values (up to 8.8 ± 0.1‰ in Chikaraishi et al. 2009; 9.7 ± 3.0‰ in Bloomfield et al. 2011). Overall, while compound-specific studies on higher TP marine animals are still limited, together these data suggest that lower $TEF_{Glu\text{-}Phe}$ values could be typical of at least mammals, birds and elasmobranchs in marine food webs. However, at the same time, the relative similarity of high values for zooplankton, invertebrates, and fish strongly suggests that $TEF_{Glu\text{-}Phe}$ values may fall into distinct groupings (Fig. 4).

[1]Zhao L (2002) Tracing amino acid metabolism of harbor seals (*Phoca vitulina*) using stable isotope techniques. PhD dissertation, University of Alaska, Fairbanks, AK

Possible explanations for variable $TEF_{Glue\text{-}Phe}$ values

There are a number of possible explanations for the Δ and $TEF_{Glu\text{-}Phe}$ differences we observed in harbor seals vs. prior work, including age and growth status, elevated TP, and overall nitrogen fractionation effects linked to nitrogen waste product. For example, the harbor seals in our study were rapidly growing pups. Prior work on bulk samples suggests that diet-to-tissue differences in δ^{15}N values are smaller in rapidly growing individuals (reviewed in Waters-Rist & Katzenberg 2010), so it is possible that trophic AAs (the dominant 'carriers' for ^{15}N-enrichment) could be less enriched in growing animals. However, the essentially identical $TEF_{Glu\text{-}Phe}$ value derived in the only prior marine mammal (adult seal) feeding experiment (Zhao 2002)[1], coupled with the similar, low TEF values for several other species (fast growing penguins in Lorrain et al. 2009), does not suggest age/growth status as an explanation for the low TEF values across several different species seen in Fig. 4.

Elevated TP itself might also contribute to differences in Δ values because of differences in both the quality and quantity of food and in particular of dietary protein. Animals with a higher TP consume diets rich in protein (i.e. high quantity), and likely have AA compositions closer to that of their own body tissues (i.e. high quality). Theory and experiment both suggest that protein quantity and quality may alter bulk δ^{15}N Δ values (e.g. Sick et al. 1997). However, the seals in this study were not particularly high in TP (~3) vs. either the fish used in prior feeding studies, or compared with wild marine animals that have been examined with the same δ^{15}N-AA approach. For example, Popp et al. (2007) showed that using previously assumed TEF values (from McClelland & Montoya 2002), when applied to high

TP wild fish (tuna; TP 4–5), returned reasonable TP estimates from δ^{15}N-AA data. Since this is currently the only high TP study available (Popp et al. 2007, Olson et al. 2010), it is difficult to rigorously evaluate the possible role of elevated TP. However, the recent feeding studies noted above also showed high $TEF_{Glu\text{-}Phe}$ values in carnivorous fish with similar TP (2–3) as our seals (Chikaraishi et al. 2009, Bloomfield et al. 2011). Therefore, while elevated TP or protein quality may well contribute to low $TEF_{Glu\text{-}Phe}$ values, taken together, it also seems somewhat unlikely as the primary factor.

Another possibility is that $TEF_{Glu\text{-}Phe}$ values could be associated with metabolic differences from ammonia vs. urea production. In animals, lighter ^{14}N is preferentially lost as either ammonia or urea, a process which enriches both the body and other N pools in ^{15}N relative to diet (e.g. Balter et al. 2006, Koch 2007). Most marine organisms, including all those on which the traditional $TEF_{Glu\text{-}Phe}$ values have been determined, produce ammonia (McClelland & Montoya 2002). In contrast, mammals and birds produce urea or uric acid, while elasmobranchs additionally retain urea as an osmolyte. A relationship of $TEF_{Glu\text{-}Phe}$ values with ammonia vs. urea excretion therefore represents another hypothesis for $TEF_{Glu\text{-}Phe}$ offsets, which would be consistent with the results from disparate organisms shown in Fig. 4 (mammals, birds, and elasmobranchs all have low $TEF_{Glu\text{-}Phe}$ values, while bony fish and other marine organisms have typically higher values). Divergences in biochemical pathways, residence times for different reservoirs, and kinetic isotopic effects between AA pools are all possible factors in $TEF_{Glu\text{-}Phe}$ differences between ammonia vs. urea excreting organisms. However, the fact that Phe δ^{15}N values remain essentially constant with trophic transfer suggests that a mechanistic explanation must focus on Δ_{Glu} values, which appear substantially smaller in urea vs. ammonia excreting organisms.

While our data cannot resolve specific biochemical mechanisms, a general consideration of the pathways for N incorporation into ammonia vs. urea suggests this hypothesis as plausible (Fig. 5), at least in marine mammals. The Glu pool is the precursor for both ammonia and urea waste (e.g. Champe & Harvey 2010); however, pathways for urea production are more complex. Formation of ammonia involves only one deamination step from Glu (Fig. 5a; via direct oxidative deamination), resulting in the direct excretion of ^{15}N-depleted ammonia, and therefore ^{15}N-enriched Glu, and corresponding large $TEF_{Glu\text{-}Phe}$ values. In contrast, urea has 2 nitrogen groups and 2 deamination steps: one nitrogen derives directly from ammonia, while the other nitrogen originates from Asp, which in turn has been derived by transamination from Glu (Fig. 5b; Champe & Harvey 2010). Thus, during urea synthesis there is an additional important intermediate pathway (Asp), which can also exchange N with broader AA metabolism. Nitrogen balance in this multi-step, multi-reservoir pathway may therefore result in urea waste that is ^{15}N enriched vs. the corresponding ammonia pathway: i.e. Glu on average may be less ^{15}N-enriched with trophic transfer because multiple biochemical pathways distribute ^{14}N more broadly throughout major biochemical compartments. Overall, a $TEF_{Glu\text{-}Phe}$ dichotomy based on urea vs. ammonia N pathways seems consistent with both our data and current literature. However, age and growth status, and elevated trophic position/or protein content could also be contributing factors—and until more direct experiments are done, must remain viable hypotheses.

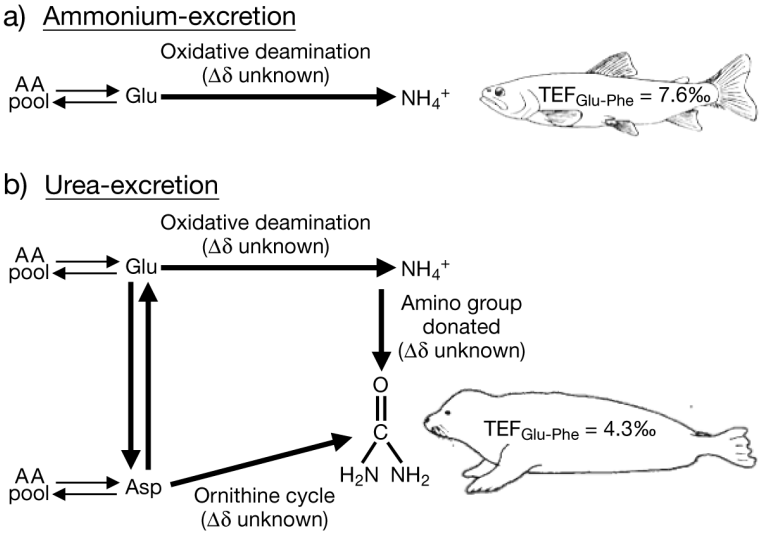

Fig. 5. Biochemical pathways for N transfer from glutamic acid to ammonia and urea. Glu pool is the precursor for N in both ammonia and urea. (a) Ammonia is produced directly through oxidative deamination of glutamate, therefore with no fractionation. Animals shown to have higher $TEF_{Glu\text{-}Phe}$ values (~7‰) to date all excrete ammonia. (b) Urea, in contrast, has 2 deamination steps; one N comes directly from Glu (via ammonia), and the other N from Asp, where the Asp N is also derived by the transamination from Glu. We hypothesize that lower $TEF_{Glu\text{-}Phe}$ values (~4.3‰) could be associated with animals using this pathway due to an equilibrium effect between Glu and Asp, dependent upon reservoir times

Implications for compound-specific isotopes of AA based trophic studies: a multi-$TEF_{Glu\text{-}Phe}$ equation for marine mammals

Several closely related calculations have been used to estimate TP of organisms using δ^{15}N-AA values (McClelland & Montoya 2002, McCarthy et al. 2007, Popp et al. 2007), differing primarily in the specific trophic and source AAs used. Some researchers have also included multiple trophic and source AAs in an effort to compensate for possible errors in any single value (McCarthy et al. 2007, Hannides et al. 2009), but in most studies only Glu and Phe have been used. Specifically, a $TEF_{Glu\text{-}Phe}$ value of 7.6 ± 0.4‰ has now been widely applied to estimate the TP of diverse animals, including marine plankton, fish, and archaeological mammals and humans (e.g. McClelland & Montoya 2002, Popp et al. 2007, Chikaraishi et al. 2009, Hannides et al. 2009). If $TEF_{Glu\text{-}Phe}$ values for some animal groups are in fact much lower, this could have a significant impact on TP estimates.

The likely validity of our lower $TEF_{Glu\text{-}Phe}$ estimates can be evaluated in several ways. First, we compared results using the widely applied $TEF_{Glu\text{-}Phe}$ value (7.6‰) on TP estimates for these harbor seals. The resulting TP estimates (average of 2.2; Table 2) are far below expectations for a marine mammal carnivore. Further, the fact that seals were fed exclusively wild-caught herring essentially rules out this result: our seals could have a TP of ~2 only if the wild herring were strictly herbivores (i.e. had TP = 1). While young herring do feed primarily on plankton, adults have a more diverse diet, including zooplankton, small fish and fish larvae (e.g. Foy & Norcross 1999). In contrast, the TP estimate of 1.8 for the herring derived from $TEF_{Glu\text{-}Phe}$ value of 7.6‰ (Table 2) corresponds very well with expected ranges, and is also consistent with the idea that currently accepted $TEF_{Glu\text{-}Phe}$ values are accurate for fish. Finally, standard bulk isotope values and scat analysis also predict the seals to be feeding at higher TP (Table 2; Germain et al. 2012). Overall, a TP near 2 seems certainly inaccurate for these seals.

We therefore derived a multi-$TEF_{Glu\text{-}Phe}$ formula to test the idea that for harbor seals (and likely at least other marine mammals), independent $TEF_{Glu\text{-}Phe}$ values must be explicitly taken into account. This approach is based on the assumption that isotopic shifts can be modeled in 2 stages (Fig. 6), each with characteristic $TEF_{Glu\text{-}Phe}$ values. The first stage represents all trophic transfers from primary production to seal prey, and also assumes that all marine prey are ammonia-excreting (which is known to be the case in our study, since seals were fed only herring), such that a $TEF_{Plankton}$ of 7.6‰ can be applied. The second stage represents only the final trophic transfer from fish to seals, for which a TEF_{Seal} of 4.3‰ is applied. Using the assumption that TEF_{Seal} applies to only one trophic transfer, a multi-$TEF_{Glu\text{-}Phe}$ trophic transfer estimate can then be derived using only measured δ^{15}N values for both Glu and Phe in seals (complete derivatization provided in the Supplement):

$$TP_{Seal} = [(\Delta^{15}N_{(Glu\text{-}Phe)Seal} - 7.7)/7.6]) + 2 \quad (5)$$

Using Eq. (5), the harbor seals in this study would then be predicted to feed at TP 2.8 (Table 2). Comparison with other possible TP estimation approaches, as well as ancillary data, suggests that this is a reasonable estimate for these seals. As noted above, if a single-TEF equation with the larger $TEF_{Glu\text{-}Phe}$ value (7.6‰) is used, then average seal TP value (2.2) would be far too low for these marine carnivores. Alternately, if we used a single-TEF equation with the smaller $TEF_{Glu\text{-}Phe}$ value from our feeding study (4.3‰), the estimated TP would be 3.2. This higher value is plausible for seals, but it would also require that the lower $TEF_{Glu\text{-}Phe}$ value (4.3‰) is in fact typical of both herring and all their lower TP marine food sources. This seems untenable based on all prior feeding studies involving both fish and plankton. Together, these comparisons strongly suggest a single $TEF_{Glu\text{-}Phe}$ value cannot be used in marine mammals. However, a multi-$TEF_{Glu\text{-}Phe}$ approach, incorpo-

Table 2. Estimated average trophic positions (TP) and ranges calculated for harbor seals *Phoca vitulina* and their food, Atlantic herring *Clupea harengus* derived from different methods. Bulk: estimated from bulk serum isotopes and varying δ^{15}N sources (Germain et al. 2012); Scat: estimated from literature, based on past harbor seal scat analyses from similar CA region (Tollit et al. 1997); Single-TEF*: using Eq. (3) and a TEF value of 7.6 originally from McClelland & Montoya (2002); Single-TEF: using Eq. (3), with our measured $TEF_{Glu\text{-}Phe}$ value of 4.3 for tested harbor seals; Multi-TEF: using Eq. (4) and $TEF_{Glu\text{-}Phe}$ values of 7.6 and 4.3 for herring and seals, respectively. Note that bulk herring TP value range is due to variation in trophic transfer values from selected source and TEF values (urea = 3.4‰, ammonia = 2.0‰; found in compilation study by Vanderklift & Posnard 2003). NA: not applicable

	Calculation method				
	Bulk	Scat	Single-TEF*	Single-TEF	Multi-TEF
TEF values	NA	NA	7.6	4.3	4.3 and 7.6
TP seals	3.3–4.3	4	2.2	3.2	2.8
TP herring	2.3–4.0	3	1.8	2.4	NA

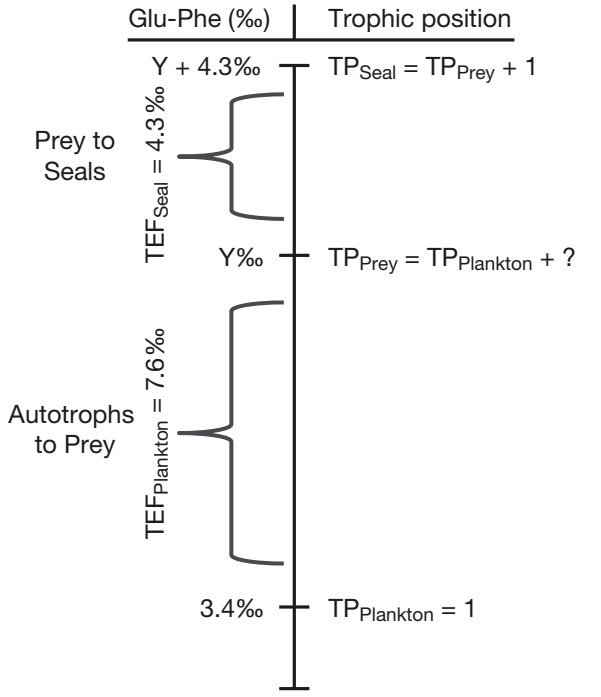

Fig. 6. Multi-TEF trophic position (TP) approach for marine mammals. Multi-TEF calculation for marine mammals tested in this study assumes a food web with 2 characteristic TEF values. All trophic transfers for prey species are modeled with a single $TEF_{Plankton}$ of 7.6‰ (based on prior data for lower TP ammonia-excreting marine animals). A single final trophic transfer from prey to seals is assumed, for which a TEF_{Seal} of 4.3‰ (value determined in this study) can be applied. At base of food web, a Glu-Phe value in autotrophs (TP = 1) is assumed to be 3.4‰ (Deniro & Epstein 1981; Minagawa & Wada 1984). Y‰ indicates Glu-Phe value final ammonia-excreting trophic step (all marine mammal prey), from which TP can be calculated directly using a standard single TEF approach. In marine mammals, Glu-Phe is therefore Y + 4.3‰, to account for an additional final low TEF trophic transfer. All TEF references indicate $TEF_{Glu\text{-}Phe}$, with subscripts shortened for simplicity

rating distinct $TEF_{Glu\text{-}Phe}$ values can provide accurate TP estimates, at least in seals.

CONCLUSIONS

This first compound-specific isotopic analysis of AA data from a controlled experimental feeding study of a marine mammal has demonstrated that while the general δ^{15}N-AA pattern in seals corresponds with past expectations, some specific AA δ^{15}N values, and in particular changes with trophic transfer, depart substantially from prior data based on lower TP marine animals. Both Pro and Thr deviate from most previous δ^{15}N-AA patterns, suggesting unique aspects to biochemical pathways for these AAs may occur in marine mammals. Major differences were also observed in Δ and TEF values for many AAs relative to expected values. In particular, the $TEF_{Glu\text{-}Phe}$ value widely used to calculate TP from compound-specific isotope AA data was found to be 4.3‰ from herring to seals—about half the typical value now commonly applied ($TEF_{Glu\text{-}Phe} \approx 7.6$‰). While compound-specific isotope AA data from higher consumers is not yet extensive, comparison to existing data in the literature strongly suggest that similar, low TEF values may also occur in other animal groups. We hypothesize that this is most likely linked to either elevated TP (increased protein content) and/or to the N-AA metabolic differences associated with ammonia vs. urea production as major N waste products. These 2 mechanisms are likely not mutually exclusive, and more extensive experiments on both high TP organisms and urea vs. ammonia excreting organisms will be required to fully understand causes of low TEF values.

Taken together, the existing data suggest that distinct but also relatively narrow ranges of $TEF_{Glu\text{-}Phe}$ exist in food webs. We therefore propose that a multi-$TEF_{Glu\text{-}Phe}$ calculation can be used (and is likely required) to accurately estimate TP, at least in marine mammals. Using a multi-$TEF_{Glu\text{-}Phe}$ approach for harbor seals yields TP estimates consistent with expected values, whereas a single-$TEF_{Glu\text{-}Phe}$ approach yields results inconsistent with both expectations and independent data. It is also important to note that the accuracy of the multi-TEF equation we propose depends on the assumption of only a single trophic step involving this low TEF value. For most marine mammals this assumption is reasonable, since the majority of marine mammal prey species (e.g. small fish and shellfish) are relatively low TP (similar to those tested in past feeding studies) and also excrete ammonia as N waste. We therefore conclude that the multi-$TEF_{Glu\text{-}Phe}$ δ^{15}N-AA approach we propose represents an important new tool for studying marine mammal ecology and trophic structure.

These results may also have significant implications for rapid expansion of compound-specific isotope AA applications beyond original work in marine planktonic systems, to mammals, terrestrial ecosystems, archaeological samples, and higher TP organisms. These data suggest that for any mammalian carnivore, TEF values will need to be carefully reexamined in order to estimate accurate TPs. For example, some recent studies on archaeological mammals and humans have suggested that compound-specific isotope AA underestimates likely TP of some speci-

mens by ~0.5 TP (Naito et al. 2010, Styring et al. 2010), a conclusion that would be consistent with the implications of this study. If the ammonia/urea dichotomy is ultimately a key factor, then compound-specific isotope AA based TP calculations in terrestrial systems (where carnivores or omnivores may have diets which include significant urea-excreting prey) may require a much more complex multi-TEF approach than presented here for marine systems. We suggest that examining TEF values in urea-excreting animals, as well as higher TP animals with varying protein content diets will need to be a key area of future research in order to realize the potential of compound-specific isotope analysis of AA for TP estimates in both marine and terrestrial food webs.

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