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ASSESSING TROPHIC ECOLOGY AND NUTRITIONAL STATUS OF MARINE MAMMALS WITH BULK AND COMPOUND-SPECIFIC AMINO ACID ISOTOPE ANALYSIS

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ABSTRACT

ASSESSING TROPHIC ECOLOGY AND NUTRITIONAL STATUS OF MARINE MAMMALS WITH BULK AND COMPOUND-SPECIFIC AMINO ACID ISOTOPE ANALYSIS

by

Leslie Roland Germain

Compound-specific isotope (CSI) analysis is rapidly growing tool in the field of ecology to assess the trophic position and foraging behavior of an animal. Only a handful of studies have examined the values and patterns of carbon and nitrogen amino acid isotopes (δ^{13}C-AA and δ^{15}N-AA), and have primarily been done on plankton and other low trophic position organisms. Since AA data separate into unique biochemical groupings, much more detailed information is revealed than the widely used bulk isotope technique - such as source of diet (offshore vs. coastal), base of the food web, trophic positions, and an animals physiology. To examine the usefulness and power of the CSI method, we tested how δ^{13}C-AA and δ^{15}N-AA patterns and values appear in several populations of harbor seals (*Phoca vitulina*) off the California coast, primarily comparing seals from San Francisco Bay (SFB) and the Channel Islands (CI).

In Chapter 1, we compared bulk isotopes in captive seals from The Marine Mammal Center to wild seal populations. Our results indicated similar trophic transfer δ^{15}N values between predator and prey compared to other organisms. It concluded that weaner seals (< 1 year) from SFB and CI had recently weaned from

their mother's milk, and were transitioning to a diet of fish and invertebrates. Chapter 2 was the very first CSI study δ^{15}N-AA on marine mammals, where we examined the 'trophic' and 'source' AA grouping differences in captive seals. A smaller trophic enrichment factor (TEF) was shown compared to past studies on plankton. We hypothesize this is attributed to an animal's main form of nitrogen excretion (ammonia vs. urea), where urea-excreting animals exhibit this lower TEF value. Thus, we propose using a new multi-TEF trophic position equation to estimate the foraging ecology of wild harbor seals (Chapter 3). This equation provides more accurate predictions, suggesting they consume prey around 2.5 to 3 trophic positions. The coupled δ^{13}C-AA and δ^{15}N-AA data suggest SFB yearlings (1 – 2 years) are nutritionally stressed, as they show higher than expected trophic position estimations. While seals in CI are consuming different types of prey (offshore vs. nearshore), but at expected trophic positions.

To

Uncle John

'Always Remembered'

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The text of this dissertation includes a reprint of the following previously published material, and one submitted manuscript:

Germain, L. R., P. L. Koch, J. T. Harvey, and M. D. McCarthy. 2011. Stable carbon and nitrogen isotopes in multiple tissues of wild and captive harbor seals (*Phoca vitulina*) off the California coast. Marine Mammal Science. DOI: 10.1111/j.1748-7692.2011.00516.x

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My contribution to all chapters was securing sources of funding, collecting harbor seal blood from captive animals, analysis of all data (methodological and statistical), and interpretation and synthesis of all data presented in these publications. The Marine Mammal Center and Jim Harvey provided harbor seal tissue samples. Matthew D. McCarthy directed and supervised the research.

Permits for collection and laboratory work are: NMFS Research Permit # 555-1565, NOAA NMFS 151408SWR2007PR00018:JGC, and CARC Mccam0803 approval.

CHAPTER 1:

Stable carbon and nitrogen isotopes in multiple tissues of wild and captive harbor seals (Phoca vitulina) off the California coast

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Stable carbon and nitrogen isotopes in multiple tissues of wild and captive harbor seals (*Phoca vitulina*) off the California coast

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ABSTRACT

Stable carbon and nitrogen isotope ratios ($\delta^{13}C$ and $\delta^{15}N$) of serum, red blood cells(RBC), muscle, and blubberwere measured in captive and wild northeast Pacific harbor seals (*Phoca vitulina richardsi*) at three coastal California sites (San Francisco Bay, Tomales Bay, and Channel Islands). Trophic discrimination factors ($\Delta_{Tissue-Diet}$) were calculated for captive seals and then applied in wild counterparts in each habitat to estimate trophic position and feeding behavior. Trophic discrimination factors for $\delta^{15}N$ of serum (+3.8‰), lipid-extracted muscle (+1.6‰), and lipid-blubber (+6.5‰) are proposed to determine trophic position. An offset between RBC and serum of +0.3‰ for $\delta^{13}C$ and −0.6‰ for $\delta^{15}N$ was observed, which is consistent with previous research. Specifically, weaner seals (<1 yr) had large offsets, suggesting strong trophic position shifts during this life stage. Isotopic values indicated an average trophic position of 3.6 at both San Francisco Bay and Tomales Bay and 4.2 at Channel Islands. Isotopic means were strongly dependent on age class and also suggested that mean diet composition varies considerably between all locations. Together, these data indicate that isotopic composition of blood fractions can be an effective approach to estimate trophic position and dietary behavior in wild pinnipeds.

Key words: stable isotopes, harbor seal, *Phoca vitulina*, carbon, ^{13}C, nitrogen, ^{15}N, San Francisco Bay, Channel Islands, Tomales Bay, trophic discrimination.

The Pacific harbor seal (*Phoca vitulina richardii*) is a small pinniped that is common on the northeastern Pacific coast in temperate and arctic waters from Baja California to the Aleutian Islands (Bigg 1981, Carretta *et al.* 2001). Harbor seals are philopatric and undertake short and shallow dives in familiar foraging locations (Stewart *et al.* 1989). Past studies using scat and stomach-gut content analyses suggested they are opportunistic feeders, whose diet mainly consists of fish and cephalopods (Antonellis and Fiscus 1980). All past studies of the diet of this species along the California coast (Harvey 1989, Torok 1994, Kopec and Harvey 1995, Grigg 2008) have used traditional methods based on scat or stomach-content analysis (Tollit *et al.* 1997). These studies offer invaluable, detailed information on prey consumption, but quantitative assessment of diet using scat or stomach-content analysis is subject to well-known biases related to differential digestion and a narrow temporal window of assessment (*i.e.*, sampling the last few meals).

Stable isotope techniques of carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) in tissues are a powerful tool for studying animal ecology, especially for organisms in difficult to observe environments (Kelly 2000, Koch 2007, Newsome *et al.* 2010). Stable isotopes provide information on trophic level, source of diet, migratory patterns, and body condition. Trophic discrimination factors are the isotopic differences between predator and prey ($\Delta^{13}C_{Tissue-Diet} = \delta^{13}C_{PredatorTissue} - \delta^{13}C_{Diet}$, and similarly for $\Delta^{15}N_{Tissue-Diet}$), and they vary with tissue type and metabolic state. Predictable ^{15}N trophic discrimination factors of $+3‰$ to $+5‰$ between prey and predator allow identification of trophic level. The average ^{13}C trophic discrimination factor is smaller and less variable in marine food webs at $+0.5‰$ to $+1.1‰$. As a consequence, $\delta^{13}C$ values in top consumers often vary with factors that affect photosynthetic fractionation of carbon isotopes at the base of the food web (*e.g.*, CO_2, growth rate, plankton size, *etc.*), allowing them to be used as a proxy of foraging location. Stable isotope analysis offers a broader temporal integration of feeding behavior, expanding the horizon of analysis to weeks, months, or even decades, depending on the type of tissue chosen for analysis (Newsome *et al.* 2010). A great potential advantage of stable isotope studies is the ability to analyze different time frames by selecting tissues with different turnover rates. Despite the broad advantages of stable isotope approaches to understanding diet, harbor seal populations have not been examined closely in the northeast Pacific, with past work conducted solely in Alaskan waters (Hobson *et al.* 1997, Burton and Koch 1999).

Zhao *et al.* (2006) determined trophic discrimination factors in *captive, adult* seals in Alaska, which were needed to assess the feeding behavior of their wild counterparts. Hobson *et al.* (1996) and Lesage *et al.* (2002) combined values from several phocids (ringed, *Pusa hispida*; harp, *Pagophilus groenlandicus*; gray, *Halichoerus grypus*; and harbor seals) for red blood cells (RBC) and serum. Both $\Delta^{13}C_{RBC-Diet}$ and $\Delta^{15}N_{RBC-Diet}$ values ranged from $+1.5‰$ to $+1.7‰$. For captive adult harbor seals ($n = 3$), Zhao *et al.* (2006) estimated a $\Delta^{13}C_{Serum-Diet}$ value of $-0.6‰$ to $+1.7‰$ and $\Delta^{15}N_{Serum-Diet}$ value of $+3.9‰$ to $+4.6‰$, and similar values have been reported for wild Alaskan pinnipeds (Hobson *et al.* 1996, Kurle and Worthy 2001).

One goal of our study was to determine if harbor seals in California (CA) have Δ values similar to their northern counterparts. In addition, ours is the first study to examine blood, muscle, and blubber tissues in *young, growing* seals. Our primary objective was to apply a dual-isotope and dual-tissue approach (RBC *vs.* serum) to assess the trophic level of different groups of harbor seals off the California coast. We analyzed isotopes in rehabilitating, stranded weaner seals (<1 yr) as well as in wild

seals in different environments in northern and southern CA (*e.g.*, a coastal site with upwelling; open water islands experiencing upwelling and the Californian Current; an urbanized, impacted bay). For wild populations, blood and tissue samples were collected in the same seasonal time frame (spring after pupping, early summer before molting) at all locations to standardize physiological shifts associated with molting, breeding, or a switch in prey that might impact isotopic values (Fadely *et al.* 1998, Zhao *et al.* 2006). Finally, examining the tropic position and diet of these animals should provide information on local environmental conditions, acting as a "tape-recorder" by documenting shifts in specific abundance of prey (kelp- or plankton-derived carbon), sources of food, weaning time frames, and trophic dynamics, while in parallel, revealing how these environmental changes affect their body conditions (nutritional stress, anabolic *vs.* catabolic metabolism).

Materials and Methods

Captive and Wild Animal Sampling

From April to June 2007, blood samples were collected from recovering harbor seal pups at The Marine Mammal Center (TMMC), a rescue and rehabilitation center in Sausalito, CA, during routine exams. Tissue samples were only collected once per individual seal, either immediately before successful release or after death. Blood serum was retained by the TMMC for successfully rehabilitated animals. Both muscle and blubber samples were collected from most deceased pups that TMMC was not able to rehabilitate, and blood serum was collected for those that fed at least 1 mo before succumbing (refer to Table S1 for time in center, tissues collected, and isotope values).

All seals were fed formula (salmon oil) for about 1 wk or until healthy enough to eat thawed, ground Atlantic herring (*Clupea harengus*). Several batches of formula and herring were collected during this timeframe, and isotopic values were pooled to establish "food" baseline values for calculation of $\Delta_{\text{Tissue}-\text{Diet}}$ values. While this study was not designed to measure tissue-turnover times, assessment of tissue isotope values across the collection suite relative to time of arrival at TMMC suggests that, as expected, serum equilibrated the fastest (by day 20), while all other tissues equilibrated more slowly (days 35–40; Fig. S1). All animal sampling procedures were approved by TMMC and UCSC Institutional Animal Care and Use Committee Protocols.

Wild seal blood samples were collected in May–June 2007 from northern and southern CA under NMFS Research Permit no. 555–1565. Males and females were sampled according to the following age classes based on their weight measurements (Bigg 1969): weaner (W), 1 mo–1 yr; yearling (Y), 1–2 yr; subadult (SA), 2–4 yr; and adults (A). All animals appeared in a healthy physical state (noticeable blubber layer and taut skin) before and after blood collection. The most northerly location, Tomales Bay (TB; 38°13.9′N, 122°58.1′W), is approximately 64 km north of San Francisco and adjacent to Point Reyes National Seashore. The site is a temperate estuary that, seasonally, receives advected water that experience oceanic and coastal upwelling. Our second northern capture site was in San Francisco Bay (SFB; 37°93.2′N, 122°41.9′W), centrally near Castro Rocks, which consist of six-rock clusters near the Richmond-San Rafael Bridge. This is the largest haul-out site in northern SFB, most likely due to being one of the few sites accessible during low tides

(Grigg 2008). SFB is an estuary-marine bay that is highly urbanized and impacted by agricultural runoff. Finally, samples were collected from seals in the Channel Islands (CI; 34°03.9′N, 120°37.4′W), in the northern islands of Santa Cruz Island, San Miguel Island, and Santa Rosa Island. The CI represent the most oceanic and least-anthropogenically disturbed location.

Blood samples were drawn from the epidural veinous sinus into nonadditive collection tubes, allowed to clot, and then centrifuged and separated into serum and RBC components, which were immediately frozen and archived at −80°C. In deceased seals, blood, pectoral muscle, and sternal axilla blubber tissue were collected during necroscopy and stored at −80°C. All samples were then lyophilized, homogenized, and stored in desiccator until ready for chemical treatment and isotopic measurements. Lipids were removed from blood (both serum and RBC), muscle, and blubber following the methods of Dobush *et al.* (1985), using petroleum ether in a Dioner Accelerated Solvent Extractor (Bannockburn, IL); samples were then dried under a fume hood for 1 h to evaporate residual solvent. For blubber samples, both the lipid-extracted material and the residual solvent containing lipids were dried and analyzed for $\delta^{13}C$ and $\delta^{15}N$ values.

Isotopic and Statistical Analysis

Nitrogen and carbon isotope analyses were conducted in the Stable Isotope Lab at the University of California, Santa Cruz on a Carlo Erba 1108 elemental analyzer (Lakewood, NJ) coupled to a ThermoFinnigan Delta Plus XP isotope ratio mass spectrometer (San Jose, CA) (EA-IRMS). Homogenized, dried RBC, serum, and tissue components were weighed into tin capsules and then combusted and analyzed on the EA-IRMS system. EA-IRMS analytical precision using the standard Pugel, after calibrating for drift of instrument and mass variability, throughout six individual runs was ±0.1‰ for carbon and nitrogen ($n = 59$). Triplicate reproducibility between 25 blood samples after standard drift corrections was ±0.03‰ for carbon and ±0.06‰ for nitrogen. Stable isotopes are reported using standard delta (δ) notation in parts per thousand (‰) using the following equation: $\delta X = [(R_{sample}/R_{standard}) - 1] \times 1{,}000$, where X is ^{13}C or ^{15}N, and R is the ratio of $^{13}C/^{12}C$ or $^{15}N/^{14}N$. The standard for carbon was Vienna PeeDee Belemnite (PDB) and the standard for nitrogen was atmospheric N_2.

Trophic discrimination factors were calculated as the difference between tissue and fish feed ± error propagation. One-way analysis of variance (ANOVA) with Tukey-Kramer's pairwise comparisons was used to compare serum and RBC mean $\delta^{13}C$ and $\delta^{15}N$ values between sex, age classes, and among the three locations. Interaction tests were performed between location and sex, age and sex, and location and age. Isotopic differences were calculated for $\delta^{13}C$ and $\delta^{15}N$ between RBC and serum ($\Delta^{13}C_{RBC-Serum} = \delta^{13}C_{RBC} - \delta^{13}C_{Serum}$, and similarly for $\Delta^{15}N_{RBC-Serum}$) for all animals, locations, and interactions between location and age. The carbon and nitrogen isotopic differences between RBC and serum were tested using paired *t*-tests for all location and age combinations.

To estimate trophic level for wild seals, we use the following equation (Pauly *et al.* 1998):

$$\text{Trophic Level} = \left[\left(\delta^{15}N_{Serum} - \delta^{15}N_{Fish}\right)/\Delta^{15}N_{Serum-Diet}\right] + \left[\left(\delta^{15}N_{Fish} - \delta^{15}N_{Plankton}\right)/\Delta^{15}N_{Fish-Diet}\right] + 1, \quad (1)$$

Table 1. Stable carbon and nitrogen isotope values (mean ± SD) in tissues of successfully released (serum) and recently deceased (muscle/blubber) harbor seals, which were under care by TMMC for more than 1 mo. Discrimination factors ($\Delta_{Tissue-Diet}$; mean ± error propagation) were determined by difference between specific tissue and diet (fish feed). LE = lipid extracted.

Tissue	n	$\delta^{13}C$	$\Delta^{13}C_{Tissue-Diet}$	$\delta^{15}N$	$\Delta^{15}N_{Tissue-Diet}$
Serum	11	−18.0 ± 0.6	+1.5 ± 0.9	16.3 ± 0.4	+3.8 ± 0.5
Muscle, LE	3	−16.8 ± 0.9	+2.6 ± 1.2	14.1 ± 0.8	+1.6 ± 1.0
Muscle, bulk	4	−17.4 ± 0.5	+2.0 ± 0.9	14.4 ± 0.9	+1.9 ± 1.1
Blubber, lipid	3	−23.0 ± 0.5	−3.5 ± 0.9	19.0 ± 1.3	+6.5 ± 1.2
Blubber, LE	4	−18.3 ± 1.0	+1.1 ± 1.0	16.7 ± 1.1	+4.2 ± 1.7
Fish feed	4	−19.4 ± 0.6		12.5 ± 0.3	

where $\Delta^{15}N_{Serum-Diet} = 3.8‰$ (Table 1) and $\Delta^{15}N_{Fish-Diet} = 3.4‰$ (Post 2002) and 1 is added as plankton represents the first trophic level.

However, since we do not know $\delta^{15}N_{Fish}$, we assume

$$\left[\left(\delta^{15}N_{Serum} - \delta^{15}N_{Fish}\right)/\Delta^{15}N_{Serum-Diet}\right] = 1, \quad (2)$$

because the transfer from seal to fish represents ~1 trophic level. Consequently,

$$\text{Trophic Level} = \left[\left(\delta^{15}N_{Fish} - \delta^{15}N_{Plankton}\right)/\Delta^{15}N_{Fish-Diet}\right] + 2. \quad (3)$$

Finally, to remove $\delta^{15}N_{Fish}$, we note that

$$\Delta^{15}N_{Serum-Diet} = \delta^{15}N_{Serum} - \delta^{15}N_{Fish} = 3.8, \quad (4)$$

which after rearrangement and substitution results in the final equation

$$\text{Trophic Level} = \left[\left(\delta^{15}N_{Serum} - 3.8\right) - \delta^{15}N_{Plankton}/3.4\right] + 2. \quad (5)$$

The $\delta^{15}N_{Plankton}$ value varies depending on location: TB, 7.5‰ (Rau *et al.* 1998); SPB, 8‰ (Cloern *et al.* 2002); CI, 6.5‰ (Germain *et al.*, unpublished data).

Results

Stranded (Rehabilitated) Seal Tissue Isotope Values and Discrimination Factors

Mean tissue $\delta^{13}C$ and $\delta^{15}N$ values, $\Delta^{13}C_{Tissue-Diet}$, and $\Delta^{15}N_{Tissue-Diet}$ values in captive harbor seals are reported in Table 1 and Table S1. Isotopic values for serum from captive animals had stabilized on the TMMC diet by 18–30 d after arrival. Muscle and blubber appeared to stabilize between 41 and 60 d (stabilization assumed from proximity of data points to level-fitted line), with an exponential decrease to a constant value by day 40 for these tissues, except for lipid-extracted muscle (LE muscle), which was still equilibrating for $\delta^{13}C$ at day 60 (Fig. S1).

Trophic discrimination factors were only estimated for individuals that had equilibrated or were likely near equilibrium. Specifically, for serum, we only used values collected more than 30 d after arrival at TMMC, whether the animal was successfully

rehabilitated ($n = 10$) or succumbed ($n = 2$). For muscle and blubber, which were only collected from animals that were euthanized, only four individuals survived in captivity for 30–60 d. Hence, our sample size is small, and these animals may still have been equilibrating to TMMC diet at the time of death. Although Table 1 reports discrimination factors for a range of different tissues and treatments (which may be useful for other studies), the key values for our study of wild harbor seals are for serum ($\Delta^{13}C_{Serum-Diet} = +1.5 \pm 0.9‰$; $\Delta^{15}N_{Serum-Diet} = +3.8 \pm 0.5‰$).

Wild Seal Tissue Isotope Values

Mean RBC and serum $\delta^{13}C$ and $\delta^{15}N$ values from 102 wild seals are reported in Table 2, along with the difference in the blood component values and the corresponding significance between RBC and serum (P-values). Over the entire data set, RBC $\delta^{13}C$ values averaged $-16.1 \pm 0.5‰$ (range, from $-14.7‰$ to $-18.4‰$), and $\delta^{15}N$ values averaged $16.6 \pm 0.7‰$ (range, 14.2‰ to 19.6‰), whereas serum $\delta^{13}C$ values averaged $-16.5 \pm 0.6‰$ (range, $-15.1‰$ to $-17.8‰$) and $\delta^{15}N$ values averaged $17.4 \pm 1.0‰$ (range, 15.8‰–20.9‰). Thus, RBC samples have greater $\Delta^{13}C$ (mean, $+0.3 \pm 0.1‰$; $P < 0.01$) and lesser $\Delta^{15}N$ values (mean, $-0.6 \pm 0.2‰$; $P < 0.01$) than the serum samples. This pattern was significant at all locations and was observed for all age/location groups with two exceptions: yearlings of TB and adults of SFB for which sample sizes were small ($n = 1$ and 4, respectively). Weaners were the only age classes that had $\Delta^{15}N_{RBC-Serum}$ values distinctly different from the mean value (Fig. 1A), whereas for $\Delta^{13}C_{RBC-Serum}$ values, all weaners and almost all SFB seals were different than the mean value (Fig. 1B). Consistent differences between the two blood components likely reflect mean differences in their amino acid composition (as different amino acids can have very different $\delta^{13}C$ and $\delta^{15}N$ values) (Macko *et al.* 1987, Kurle 2002). The differences in Δ values observed among age/sex/location groups may reflect the fact that these blood components have different turnover rates and thus, record slightly different time periods.

Because this is a relatively large data set with several variables (age, sex, location), we only report statistically significant observations. RBC and serum generally showed consistent trends compared to each other, although there were a few discrepancies. Mean location $\delta^{13}C$ values varied <1‰ and were highest for TB and lowest for SFB (Table 2, Fig. 2). Mean trophic position varied by 0.6 among locations, mostly attributed to weaners. Trophic position for adults, subadults, and yearlings varied by 0.2–0.3 within locations (TB 3.5–3.7, SFB 3.2–3.5, CI 3.9–4.2, $P < 0.01$, Table S2). At SFB and CI, weaners had the greatest $\delta^{15}N$ values (with trophic positions of 4.0 and 4.7, respectively), whereas yearlings had the lowest (with trophic positions of 3.4 and 3.9, respectively) (Table 2, Fig. 2, S2). There were significant isotopic interactions between age and location, and location and sex, depending on the blood fraction (ANOVA; significant interactions in bold in Table S2).

The interaction of $\delta^{15}N$ and $\delta^{13}C$ between age and location was significant ($P < 0.01$). In the RBC fraction, TB had the lowest $\delta^{15}N$ across age classes (the difference in $\delta^{15}N$ between age class and TB and CI were statistically significant and had $P < 0.01$, Table 2, and Fig. 3A), and yearlings had the lowest values among age classes (~15.0‰ compared to 16.0‰–17.0‰ for other age groups). At a given site, the most ^{15}N-enriched values always occurred in weaners, with an increase in trophic position by ~0.3 (or ~1.3‰), relative to adults. Serum $\delta^{15}N$ values at CI and SFB had significant differences between age classes ($P < 0.01$, Fig. 3B). CI exhibited a

Table 2. Wild harbor seal $\delta^{13}C$ and $\delta^{15}N$ (mean ± SD) for each location for all age groups (ALL), interactions between locations and age groups, and the total mean of all 102 seals located in the final row. Sex (no. of F, no. of M) refers to the number of females and males in each category. Location and age abbreviations found in text (section Methods). $\Delta_{RBC-Serum}$ reports the difference between RBC and serum blood fractions for both $\delta^{13}C$ and $\delta^{15}N$ (‰), while significant differences are in bold. *P*-values of $\Delta_{RBC-Serum}$ are displayed using matched pairs analysis, where significant differences exist when $P < 0.05$. Bolded $\Delta_{RBC-Serum}$ values are when both the value is greater than the mean and significant.

		Sex no. of F,	RBC		Serum		$\Delta_{RBC-Serum}$		$\Delta_{RBC-Serum}$		Trophic
Location	Age	no. of M	$\delta^{13}C$ (‰)	$\delta^{15}N$ (‰)	$\delta^{13}C$ (‰)	$\delta^{15}N$ (‰)	$\delta^{13}C$ (‰)	*P*	$\delta^{15}N$ (‰)	*P*	level[a]
TB	ALL	24, 11	−15.9 ± 0.5	16.3 ± 0.6	−16.2 ± 0.7	16.9 ± 0.6	0.3	<0.01	−0.5	<0.01	3.6
	A	13, 6	−15.8 ± 0.5	16.5 ± 0.5	−16.1 ± 0.7	17.0 ± 0.6	0.3	0.04	−0.5	<0.01	3.7
	SA	8, 5	−16.0 ± 0.5	16.1 ± 0.3	−16.4 ± 0.5	16.7 ± 0.5	0.4	<0.01	−0.6	<0.01	3.6
	Y	2, 0	−16.4 ± 0.2	15.0 ± 1.1	−17.1 ± 0.5	16.3 ± 0.1	0.8	0.13	−1.3	0.37	3.5
	W	1, 0	−16.2	16.7	−15.6	16.3	−0.5	–	0.4	–	3.5
SFB	ALL	9, 8	−16.5 ± 0.7	16.8 ± 1.2	−16.9 ± 0.7	17.4 ± 1.3	0.5	<0.01	−0.7	<0.01	3.6
	A	3, 1	−16.7 ± 1.2	16.6 ± 1.6	−15.9 ± 0.2	16.0 ± 0.2	−0.7	0.22	0.7	0.02	3.2
	SA	2, 1	−16.5 ± 1.0	16.4 ± 0.7	−17.0 ± 0.2	16.8 ± 0.6	0.5	0.45	−0.4	0.02	3.5
	Y	1, 2	−16.3 ± 0.6	15.7 ± 0.1	−16.4 ± 0.8	16.4 ± 0.5	0.1	0.32	−0.7	0.11	3.4
	W	3, 4	−16.5 ± 0.3	17.6 ± 0.9	−17.4 ± 0.4	18.7 ± 0.8	1.0	<0.01	−1.0	<0.01	4.0
CI	ALL	29, 23	−16.1 ± 0.4	16.8 ± 0.5	−16.5 ± 0.4	17.9 ± 1.0	0.4	<0.01	−1.0	<0.01	4.2
	A	17, 13	−16.0 ± 0.4	16.8 ± 0.5	−16.4 ± 0.3	17.7 ± 0.6	0.3	<0.01	−0.9	<0.01	4.2
	SA	6, 4	−16.1 ± 0.4	16.7 ± 0.4	−16.6 ± 0.3	17.5 ± 0.5	0.5	<0.01	−0.8	<0.01	4.1
	Y	1, 2	−16.6 ± 0.1	16.0 ± 0.2	−16.9 ± 0.2	16.6 ± 0.6	0.3	0.19	−0.6	0.11	3.9
	W	5, 4	−16.0 ± 0.7	17.4 ± 0.3	−16.8 ± 0.7	19.5 ± 0.9	0.8	<0.01	−2.1	<0.01	4.7
Mean	ALL	62, 40	−16.1 ± 0.5	16.6 ± 0.7	−16.5 ± 0.6	17.4 ± 1.0	0.3 ± 0.1	<0.01	−0.6 ± 0.2	<0.01	3.8

[a]Trophic level = $[(\delta^{15}N_{Serum} - 3.8) - \delta^{15}N_{Plankton}/3.4] + 2$ using $\delta^{15}N_{Plankton}$ values of TB = 7.5‰, SFB = 8‰ and CI = 6.5‰, described in Methods.

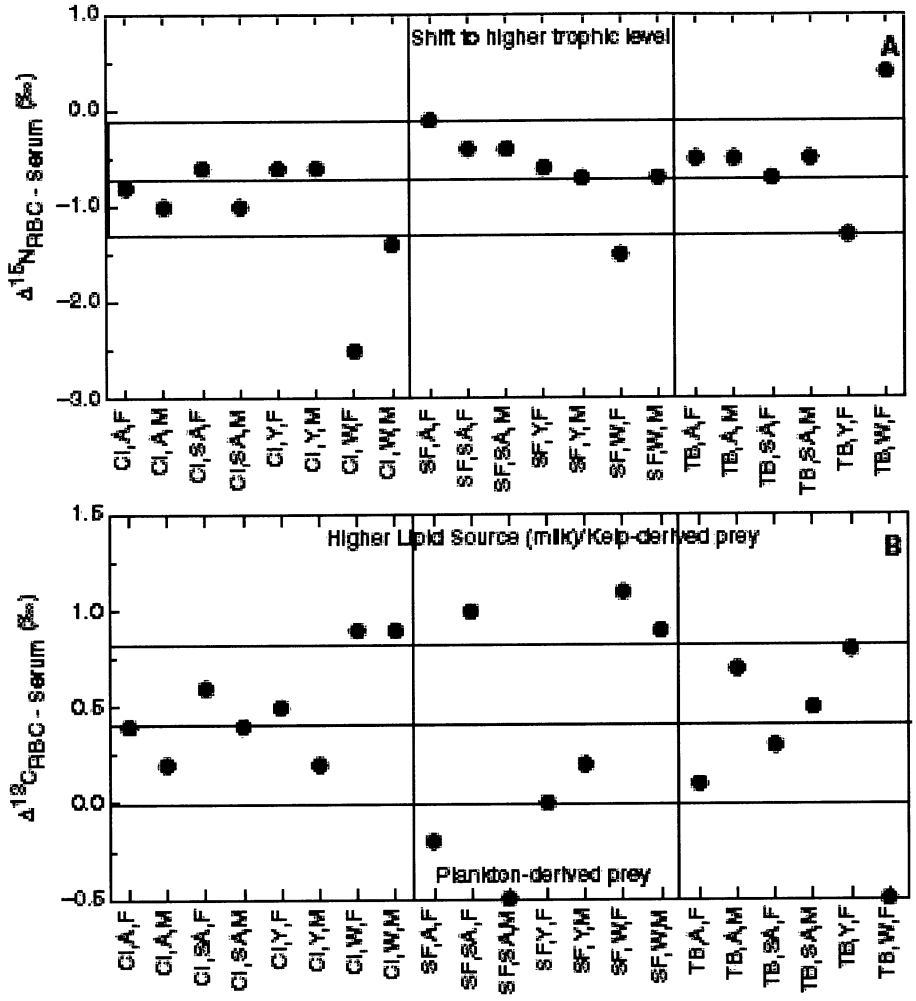

Figure 1. The difference in isotopic value between RBC and serum ($\Delta_{RBC-Serum}$) for (A) nitrogen and (B) carbon for each seal interaction (location and age and sex). The line is the mean difference, where the shaded area represents the SD for total error propagation between both blood fractions (0.4‰ for $\delta^{13}C$ and 0.5‰ for $\delta^{15}N$). Location and age abbreviations defined in text.

similar pattern to the RBC fraction, whereas SFB had greater weaner $\delta^{15}N$ values and relatively similar values among other age classes, and TB had a minor increase with age (though we have data for only one weaner).

For $\delta^{13}C$ values, the patterns in the RBC fraction were similar at TB and CI; weaners were slightly higher than yearlings, then values increased again from yearlings to ^{13}C-enriched adults, ($P > 0.01$ for age and location interaction, Fig. 3C). The $\delta^{13}C$ values in SFB were different from the other two sites. Values of $\delta^{13}C$ were lowest in weaners and decreased from younger adults to adult animals (Fig. 3C). In the serum fraction, TB and CI had a progressive increase in $\delta^{13}C$ values of

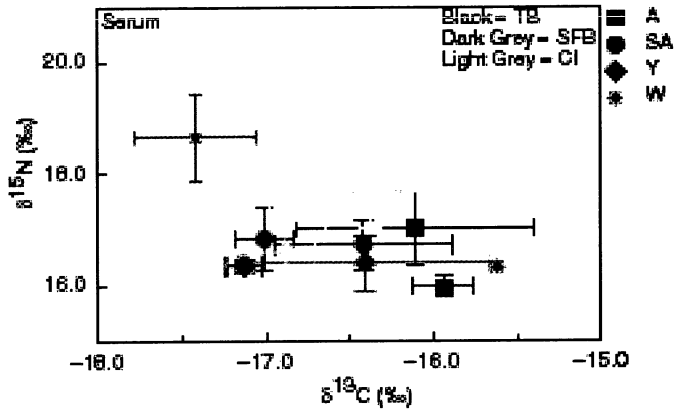

Figure 2. The means ± SD of $\delta^{13}C$ vs. $\delta^{15}N$ in serum of the four age classes (A, SA, Y, W) for each location (TB, SFB, CI). Location and age abbreviations defined in text. TB = black, SFB = dark gray, CI = light gray. A = squares, SA = circles, Y = diamonds, W = stars.

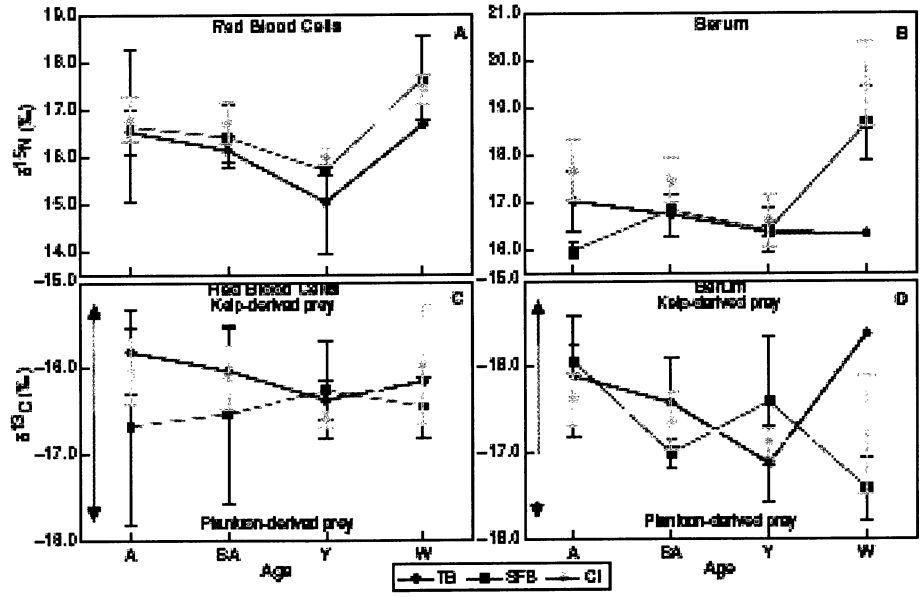

Figure 3. Significant interactions ($P < 0.05$, Table S2) between location and age for $\delta^{15}N$ (mean ± SD) in (A) RBC and (B) serum and for $\delta^{13}C$ (mean ± SD) in (C) RBC and (D) serum. As $\delta^{13}C$ approaches −10‰, it resembles kelp-derived carbon sources, whereas approaching −20‰ resembles plankton-derived. Location and age acronyms defined in text. TB = black diamonds, SFB = dark gray squares, CI = light gray circles.

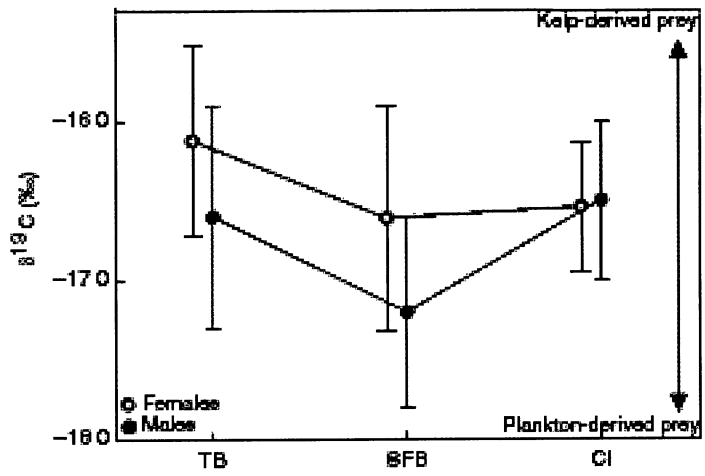

Figure 4. The significant interaction of mean $\delta^{13}C \pm SD$ values ($P < 0.05$, Table S2) between location and sex in serum. Since RBC displayed a consistent offset to serum at all locations ($\Delta_{RBC-Serum} \leq 0.3‰$), we only show serum. As $\delta^{13}C$ approaches −10‰, it resembles kelp-derived carbon sources, whereas approaching −20‰ resembles plankton-derived. Female = open symbols, Male = closed symbols.

yearlings to adult seals, consistent with RBC data (CI had $P < 0.05$ between age classes). SFB was again different, as the trend in serum was not consistent, with large swings between age classes (SFB had $P < 0.01$). For weaners, there was no consistent pattern of significant difference relative to other age classes among all three sites. (Fig. 3D).

For both blood components, an interaction of location and sex contributed significantly to differences in $\delta^{13}C$ values. CI and TB seals have similar $\delta^{13}C$ values for each sex, in both serum and RBC, whereas SFB seals were always depleted in ^{13}C compared to other locations (Fig. 4). The ^{13}C-depletion in SFB seals relative to seals compared to the other sites ($P < 0.05$) is greatest in males (−0.9‰ for RBC and −0.6‰ for serum) whereas ^{13}C-depletion in SFB females is much less (−0.1‰ for RBC and −0.4‰ for serum).

Discussion

Tissue Discrimination Factors from Stranded Seals

To date, only three controlled feeding studies have examined Δ values in harbor seals (Hobson *et al.* 1996, Lesage *et al.* 2002, Zhao *et al.* 2006), and all reported exclusively on *adults*, examining blood, hair, whiskers, or nails. We examined serum in growing, successfully rehabilitated pups that had been at TMMC for more than a month. In addition, we analyzed blubber and muscle tissue, from a small number of animals that were considered nutritionally healthy (*i.e.*, eating satisfactory proportions of fish feed for more than a month), but were unable to be released for a variety of physiological reasons (trauma, neurological, colitis, enteritis) and were euthanized (Table S1).

Isotopic incorporation into tissues occurs during growth and tissue breakdown and resynthesis (*i.e.*, turnover). It takes ~3 isotopic half-lives for a tissue to equilibrate fully to a new prey after a switch in diet. Tissues undergoing greater metabolic activity (serum, liver, fat) have shorter half-lives than tissues with lesser metabolic rates (brain, muscle, bone) (Tieszen *et al.* 1983). For example, plasma from American black bears (*Ursus americanus*) had a half-life of 4 d and fully incorporated isotopes from an altered diet within 10 d (Hilderbrand *et al.* 1996), and liver from gerbils had a similar half-life of 6.4 d (Tieszen *et al.* 1983). Previous work has indicated that isotopic equilibrium for seal serum occurs over similar time frame (Kurle 2002, Zhao 2002). Zhao *et al.* (2006) concluded that due to the turnover rate of serum, isotope values were able to record a shift in diet of less than 1 mo. In addition, younger, rapidly growing animals have a higher metabolic and tissue turnover rate from extremely active anabolism (Sakano *et al.* 2005). Thus, we are confident that serum had equilibrated after 30 d on TMMC rations and, therefore, that our Δ values are robust.

Our $\Delta^{15}N_{Serum-Diet}$ values of $+3.8 \pm 0.5‰$ and $\Delta^{13}C_{Serum-Diet}$ values of $+1.5 \pm 0.9‰$ are quite similar to trophic discrimination factors from studies of other phocids studies, where $\Delta^{15}N_{Serum-Diet}$ ranged from $+1.7‰$ to $+4.6‰$ and $\Delta^{13}C_{Serum-Diet}$ ranged from $-0.6‰$ to $+1.7‰$ (Hobson *et al.* 1996, Lesage *et al.* 2002, Zhao *et al.* 2006). $\Delta_{Tissue-Diet}$ values may vary with the macromolecular composition of diet or with the growth state of the animal. Trueman *et al.* (2005) found lower $\Delta^{15}N$ values in salmon undergoing intensive growth than those experiencing little to no growth, with no discernable impact on $\Delta^{13}C$ values. However, studies on rodents indicated that less than 10% of isotopic change is attributed to growth (West *et al.* 2001, MacAvoy *et al.* 2005). Other factors, such as nutritional stress and protein level of diet also can affect $\Delta_{Tissue-Diet}$ values (Sick *et al.* 1997, Zhao 2002). When animals ingest protein for calories at levels beyond that needed for maintaining nitrogen balance, they produce more nitrogenous waste (*e.g.*, urea), which is ^{15}N-depleted relative to body tissues and serum. As a consequence, $\delta^{15}N$ values are higher in serum, RBC, and body tissues than on low-protein diets (Sick *et al.* 1997). Correspondingly, higher protein diets (pollock) increase $\Delta^{15}N_{Serum-Diet}$ *vs.* lower protein diets, such as herring ($4.6‰$ *vs.* $3.9‰$; Zhao *et al.* 2006). For optimum growth, rehabilitating seals at TMMC were fed a mixture of ground or whole herring, which is a high-lipid/high-energy density fish, resulting in a mean $\Delta^{15}N_{Serum-Diet}$ value similar to that for herring in Zhao *et al.* (2006). For $\Delta^{13}C_{Tissue-Diet}$, because lipids are strongly ^{13}C-depleted, high-lipid diets are expected to yield lower $\Delta^{13}C_{Serum-Diet}$ values than low-lipid diets and may even be negative relatively. The similarity of our values with those previously reported for North Atlantic harbor seals also consuming herring ($\Delta^{15}N_{Serum-Diet} = +3.5‰$ and $\Delta^{13}C_{Serum-Diet} = +1.6‰$ after lipid corrections; Lesage *et al.* 2002) indicates that these values are relatively invariant when fed a high-lipid/high-energy prey. Because harbor seals require both high fat and high energy to sustain a long-term, reproductive life, we recommend using $\Delta^{15}N_{Serum-Diet}$ of $+3.8‰$ and $\Delta^{13}C_{Serum-Diet}$ of $+1.5‰$ to interpret wild trophic position in healthy populations.

Blubber is comprised of connective tissue and fat/lipid, where lipids are obtained from both diet and biosynthesis. For blubber, the $\Delta^{13}C_{Lipid-Diet}$ was $-3.5 \pm 0.9‰$. This is not surprising; lipids are often the most ^{13}C-depleted compound class in animals by $5‰$ to $10‰$ (DeNiro and Epstein 1977). After lipid-extraction, the $\Delta^{15}N_{Blubber-Diet}$ value for the residual lipid material (*e.g.*, blubber) was $+6.5 \pm 1.2‰$. However, the $\Delta^{15}N_{LE\ Blubber-Diet}$ component had a smaller value similar to the $\Delta^{15}N_{Serum-Diet}$. The $\Delta^{15}N$ differences between blubber samples could be related

to nitrogen composition, where the LE blubber fraction has very little nitrogen, less than half a percent, mostly likely as small enzymes or small polypeptide fragments derived from connective tissue, possibly affecting instrument analytical precision.

Although we believe serum and blubber had adequate times for isotopic equilibration to TMMC diet, lipid-extracted muscle $\delta^{13}C$ values had not stabilized, even for samples collected 60 d after the diet switch, whereas $\delta^{15}N$ values had stabilized. The $\delta^{13}C$ values only changed by $-0.5‰$ between days 20–40 ($n = 5$), yet decreased an additional $1.5‰$ by day 60 ($n = 1$; Fig. S1). The $\Delta^{13}C_{LE,Muscle-Diet}$ value was $+2.6 \pm 1.2‰$, within error propagation of serum. The $\Delta^{15}N_{LE,Muscle-Diet}$ value was $+1.6 \pm 1.0‰$, however, slightly less than the value of $+2.4‰$ reported for another phocid ($n = 1$ harp seal; Hobson *et al.* 1996). This difference might be attributed to the age of the seals analyzed. Only weaners were sampled in this study, and these animals were growing rapidly, possibly reducing their flux of ^{15}N-depleted waste urine to form muscle tissue and to maintain higher rates of protein synthesis and catabolism. Regardless, a $\Delta^{15}N_{LE,Muscle-Diet}$ value of $+2.0‰$ appears to be a reasonable value for assessing trophic information in wild phocid populations.

Wild Seal Tissue Isotope Values

Stable isotope analysis has been used to assess the diets of pinnipeds off CA, Oregon, and Washington (Hobson *et al.* 1997, Hobson and Sease 1998, Burton and Koch 1999, Burton *et al.* 2001, Hirons *et al.* 2001, Kurle and Worthy 2001, Hobson *et al.* 2004, Aurioles *et al.* 2006, Newsome *et al.* 2006, Kurle and Gudmundson 2007). Yet, there are few isotopic studies of harbor seals from the northeast Pacific, and these were on the Bering Sea and Gulf of Alaska (Burton and Koch 1999, Hirons *et al.* 2001). Comparisons to seal populations in central and southern CA may be particularly useful in understanding harbor seal ecology in highly urbanized areas such as SFB, where animals may have to travel greater distances to obtain adequate nutrition due to the poor quality or quantity of prey.[1]

The significant differences in mean $\delta^{15}N$ values among sites (TB > $0.5‰$ to SFB in serum, and TB > $1‰$ to CI in serum; Table 2) may reflect differences in $\delta^{15}N$ values at the base of the food web or differences in trophic structure. Planktonic $\delta^{15}N$ values vary depending on the source of nitrogen to the oceanic system and the extent of uptake. For example, if the massive deep ocean nitrate pool ($\delta^{15}N \sim +5‰$) is the source of nitrate in the photic zone (and if this nitrate is completely exhausted by planktonic uptake), then plankton will have a value similar to the pool. In highly productive regions (with suboxic waters at depth), denitrification can enrich the ^{15}N in the upwelled nitrate to $\sim 15‰$ to $20‰$, strongly enriching plankton (Altabet *et al.* 1999). Freshwater runoff also has ^{15}N-enriched nitrate in urbanized areas. In areas or seasons where other nutrients limit production, phytoplankton can be ^{15}N-depleted relative to the nitrate pool due to incomplete nitrate utilization. Finally, as zooplankton and fish are typically ^{15}N-enriched by $3‰$ to $4‰$ with each trophic transfer, longer food webs have greater $\delta^{15}N$ values in top consumers (*e.g.*, Monroya 2007).

To calculate trophic level for each location and age class, $\delta^{15}N$ values at the base of the food web were assessed using values for particulate nitrogen from the literature (described in Methods). TB, a temperate estuary with coastal upwelling, obtains

[1]Personal communication from California Department of Fish and Game, Bay-Delta Region, 4001 N. Wilson Way, Stockton, CA, May 2009.

its nutrients primarily from suboxic and deep nitrate entrained in the California Current, thus, its plankton values are about +7.5‰ (Rau *et al.* 1998). In SFB, an urbanized estuary highly influenced by terrestrial runoff, particulate $\delta^{15}N$ values averaged +8.0‰, ranging from 5.0‰ to 10.6‰ due to variations in freshwater and tidal inputs (Cloern *et al.* 2002, Huntington and Boyer 2008). CI, the most oceanic of all the sites, are strongly influenced by the California Current and seasonal upwelling. It has the lowest $\delta^{15}N$ values averaging about 6.5‰ (Germain *et al.*, unpublished data, samples collected in 1999), but values ranging from 6.9‰ to 8‰ were reported for the late 1980s (Altabet *et al.* 1999). Given these baseline $\delta^{15}N$ values, harbor seals fed at trophic levels ranging from 3.2 to 4.7, averaging 3.6 in TB and SFB and 4.2 in CI (Fig. S2). This suggests seals in the CI consume higher trophic level organisms, presumably feeding on more open-ocean than coastal prey (*i.e.*, squid, larger fish). Open-ocean fish tend to feed on longer food webs, where phytoplankton are the base of the food web, followed by microzooplankton, macrozooplankton, small fish, medium fish, large fish, then seals, resulting in an overall greater $\delta^{15}N$ value. In coastal systems, the food web is shorter, generally going from plankton to zooplankton to medium-sized fish to seals (*e.g.*, Monroya 2007).

Changes in habitat, food sources, metabolic, and physiological parameters affect stable carbon isotope ratios. The $\delta^{13}C$ data support the inferences above trophic level, assuming source $\delta^{13}C$ values of plankton range from −19‰ to −22‰ in CI and TB (Rau *et al.* 1998), and values of −17‰ to −27‰ in SFB (Cloern *et al.* 2002). These values are mainly influenced by changes in carbon source (*i.e.*, kelp-based *vs.* plankton-based prey). Kelp $\delta^{13}C$ values (−12‰ to −14‰) are much higher than those found in plankton (Page *et al.* 2008). Therefore, if a seal was feeding at a trophic position of 4.0 relative to plankton-derived prey, its $\delta^{13}C$ values would be about −16‰ to −19‰, similar to what was measured (Fig. 2). If feeding on eelgrass or kelp-derived prey, instead of plankton-derived, we would expect much higher $\delta^{13}C$ values of −5‰ to −11‰ (TB eelgrass ranges from −8‰ to −10‰ and CI kelp from −12‰ to −14‰; Fourqurean *et al.* 1997, Page *et al.* 2008). Overall, harbor seals from all coastal CA locations are feeding at similar trophic levels, preferring to consume prey around a trophic position of 3.

The differences between age classes for each location is particularly noteworthy, especially the weaners from SFB and CI, who are ^{15}N-enriched relative to the adults and subadults (Table 2, Fig. 2). The weaners in these locations are still obtaining the majority of their nutrition from their mother's milk and are ^{15}N-enriched by about 0.7 trophic level. Pups nurse for approximately 1 mo before they begin capturing and feeding on crustaceans and fish. Because they are unable to swim to the depths and distances of their older counterparts, they are restricted to feeding on prey that is nearby and easily obtained, making them susceptible to any potential food deficiencies within their constrained home range. Yearlings consume at the lowest trophic level, and while not drastically lower than the older seals, they are consistently lower by 0.2, presuming they feed on smaller prey (benthic, crustaceans, anchovies). In contrast, the seals in TB only vary in trophic position by 0.2 among all age classes, indicating all seals consumed similar prey items.

In this study, we determined a $\Delta^{15}N_{RBC-Serum}$ of -0.6 ± 0.2‰ for wild seals (Table 2), which is related to differences in amino acid compositions, and trophic level calculations varied less than 0.3 for all categories, except TB weaners and SFB adults. When $\Delta^{15}N_{RBC-Serum}$ values for an age/sex/location group fall within the range of error propagation, it suggests that any differences between the blood fractions are due to biochemical pathways. However, in several cases, the $\Delta^{15}N_{RBC-Serum}$ value

was outside of this range (ourside of gray area, Fig. 1A), indicating a change in trophic level over the different time frames represented by these tissues (weeks for serum, months for RBC). At all locations, the weaners had a shift in diet. Specifically CI and SFB weaners switched from lower trophic level (indicated by RBC values) to higher trophic level (indicated by serum values), obtaining their entire diet from their mother's milk compared with *in utero*. This implies the isotopic incorporation experienced *in utero* is similar to that for other maternal tissues (including milk protein), whereas a weaner consuming these tissues as milk is a trophic level higher. The opposite trend was observed in the single weaner sampled in TB. This animal had likely transitioned from mother's milk to the lower trophic-level prey consumed by yearlings, since it had a serum $\delta^{15}N$ value identical to yearlings from TB (Table 2).

Carbon differences mainly arise from a change in the source of diet, whether a shift from milk to prey, a shift from open-ocean to coastal prey, or a shift from kelp-derived to plankton-derived prey. Once again, most $\Delta^{13}C_{RBC-Serum}$ values fall within the propagation of error (Fig. 1B), except for the weaners and almost all the seals in SFB (excluding the yearlings). The weaners in CI and SFB had positive $\Delta^{13}C_{RBC-Serum}$, implying their current diet was composed of lipid-rich milk (with a lower $\delta^{13}C$), whereas the TB weaner had likely transitioned to a fish and invertebrate diet (though its $\delta^{13}C$ value did not match that of yearlings). The differences in adults and subadults in SFB were likely caused by shifts in prey among benthic or pelagic sources. Harbor seals tend to forage within 5 km of their place of birth, but a small percentage are thought to travel further (Lander *et al.* 2002), which suggests either greater variability or lesser density of prey in SFB prey. It is possible these older seals are venturing further distances outside of SFB to obtain adequate nutrition, which has been observed by individual harbor seals preferring particular foraging and habitat ranges (*i.e.*, more open-ocean; Nickel 2003).

Significant Interactions between Location and Age Class and Location and Sex

The most significant $\delta^{15}N$ interactions existed between locations and age classes (Fig. 3). It is important to note the striking similarities among these interactions from RBC, whereas the patterns in serum differ among location, confirming the value of obtaining tissues recording different timeframes. Location isotopic values were dependent upon seal age class, especially in the serum fraction (Table S2). In RBC $\delta^{15}N$ values, the pattern is consistent at all locations, with highest values in weaners, intermediate values in adults to subadults, and the lowest values in yearlings (Fig. 3A). This suggests that the yearling seals are feeding at the lowest trophic levels, and that seals begin feeding on higher level organisms as they mature (Oates 2005). In serum, this pattern still holds true for CI, but TB exhibited comparable $\delta^{15}N$ values of ~16‰–17‰ for all age classes, with *lowest* values and, thus, trophic feeding found in the single weaner (Fig. 3B). The weaners in SFB and CI, however, were the most ^{15}N-enriched, suggesting weaners have yet to wean completely from their mother's milk. SFB weaners are ^{15}N-enriched relative to the adults by ~2.5‰ (rather than a full trophic step of ~3.8‰), which implies they recently fed on prey instead of milk. CI weaners are greater in $\delta^{15}N$ by ~2.0‰ than the adults, and they are likely in the process of tissue equilibration to their diet (Fig. 3B). The low $\delta^{15}N$ value for adult SFB seals relative to serum from other adults and subadults indicates that they recently consumed lower trophic-level prey (crab, anchovies).

With respect to $\delta^{13}C$ interaction effects, RBC patterns in TB and CI follow each other closely, with yearlings exhibiting the lowest $\delta^{13}C$ values, but they only differentiate by 0.6‰ overall (Fig. 3C). However, yearlings in SFB have greater

$\delta^{13}C$ compared with other age classes, possibly due to a greater proportion of their diet coming from kelp-derived prey *vs.* plankton-derived prey. Once again, serum had greater variability among age classes (1.5‰ overall), with TB following the RBC pattern (Fig. 3D). Serum from CI seals barely shows any isotopic differences, indicating all age classes are all feeding on prey derived from similar sources. Both adults and yearlings in SFB are ^{13}C-enriched by about +1.0‰ compared with the subadults and weaners, which confirms the conclusion by ^{15}N of adults consuming more benthic/kelp-derived organisms than other locations (Page *et al.* 2008). Similar source and trophic interactions are most likely the main reason for age class differences in minimally disturbed locations such as TB and CI, where SFB is more influenced by allochthonous sources (*i.e.*, coastal *vs.* open-ocean).

Another significant $\delta^{13}C$ difference was the interaction between location and sex for both RBC and serum (Table S2, Fig. 4). Since RBC displayed a consistent offset to serum at all locations ($\Delta_{RBC-Serum} \leq 0.3$‰), we only describe the serum fraction. It appears females are physically constrained in both bays (SFB and TB), probably due to obligatory proximity to weaners, whereas the males can venture to more distant locations to consume higher-lipid/higher-energy prey (lower $\delta^{13}C$ values). Male and female seals in CI feed essentially on similar prey organisms, as they exhibit comparable $\delta^{13}C$ values.

Conclusions

In summary, blubber, muscle, and other tissue $\Delta_{Tissue-Diet}$ values still need more comprehensive analysis for pinnipeds, especially from animals fed for a longer period on a consistent diet, since we were unable to collect such data from our short-term study of captive mammals under marine mammal protocols. Our $\Delta_{Tissue-Diet}$ values will be useful in interpreting long-term isotopic records of wild seals in light of trophic-level changes or shifts in location. For future work, we recommend using a robust multispecies approach to investigate trophic structure, with $\Delta^{15}N_{Tissue-Diet}$ value of +3.8‰ and +2.0‰ for serum and muscle, respectively. Harbor seals along coastal CA are feeding at varying trophic levels, ranging from 3.6 to 4.2, mostly impacted by age. Weaners are still equilibrating isotopically after consuming mother's milk and yearlings are feeding at a slightly lower trophic position than adults and subadults in spring. Also, this study stresses the value of obtaining tissues that record different time frames to evaluate shifts in diet and trophic structure. Future studies with compound-specific amino acid isotope analysis will allow researchers to simultaneously determine $\delta^{15}N$ and $\delta^{13}C$ values at the base of food web and exact trophic level of the forager. This more detailed compound-specific isotope analysis may be a powerful tool to assess nutritional status in marine mammals, especially seals under extreme anabolic or catabolic conditions.

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Supporting Information

The following supporting information is available for this article online:

Figure S1. $\delta^{13}C$ and $\delta^{15}N$ equilibration times for serum, muscle (Bulk and Lipid-extracted [LE]), and blubber (Lipid and LE). Each data point represents an individual seal, which had either died or was successfully released between days 0 to 93 from admittance date to TMMC (refer to Table S1). Seals were fed salmon oil ($\delta^{13}C = -21.7‰$, $\delta^{15}N = 5.4‰$) for the first week, and then switched to ground herring for the remainder of time ($\delta^{13}C = -19.4‰$, $\delta^{15}N = 12.5‰$).

Figure S2. Stable carbon vs. nitrogen in blood serum of harbor seals for all locations. Refer to legend for symbol description. TB = Tomales Bay (black), SFB = San Francisco Bay (dark gray), CI = Channel Islands (light gray), A = Adult > 4 yr (diamond), SA = Subadult 2–4 yr (square), Y = Yearling 1–2 yr (star), W = Weaner 1 mo–1 yr (triangle).

Table S1. $\delta^{13}C$ and $\delta^{15}N$ of all tissues (serum, muscle, blubber) for captive, rehabilitating seals at TMMC (LE = Lipid Extracted). Tissue was collected once, on final day in center either due to successful release, death, or euthanization (R, D, E). Discrimination factors ($\Delta_{Tissue-Diet}$) were determined from seals at TMMC for greater than 30 d (bolded). Health (1 = emaciated, 7 = obese). Refer to Table S2 for specific tissue isotopic equilibrium graphs after grouping values from seals released or deceased over varying time frames in TMMC.

Table S2. ANOVA statistical analysis of interaction effects between sex, location, and age were used for both cell fractions (RBC and serum) on $\delta^{13}C$ and $\delta^{15}N$. Degrees of freedom (df), sum of squares, *F*-Ratios, and *P*-values are given for each test. Significant interactions are those with $P < 0.05$ (bolded) and are presented in Figures 3 and 4. Both RBC and serum are discussed to determine temporal changes in trophic-level feeding behavior in specific groupings.

CHAPTER 2:

Nitrogen isotope fractionation in amino acids from harbor seals: Implications for estimating trophic relationships in urea vs. ammonia excreting animals

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ABSTRACT

Compound-specific isotope analysis of individual amino acids is a rapidly growing tool in ecological studies to assess diet and trophic position (TP) in both modern and paleo foodwebs. This approach depends on understanding both the pattern of nitrogen isotope ($\delta^{15}N$) values in amino acids (AA), and in particular $\delta^{15}N$ changes for specific AAs with trophic transfer. We conducted the first controlled feeding study examining $\delta^{15}N$ values in AAs in a marine mammal (harbor seals, *Phoca vitulina*, fed exclusively on herring, *Clupea harengus*). The pattern of $\delta^{15}N$ variation among AAs in seals was similar to that observed in other heterotrophs. 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 currently broadly used for TP estimation ($TEF_{Glu\text{-}Phe}$; the expected difference in $\delta^{15}N$ values between glutamate vs. phenylalanine per trophic step) 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 elasmobranches (skates) suggest that similar, low TEF values may also be characteristic of these taxa. Together, these data imply that urea-producing animals have different, but also diagnostic, changes in $\delta^{15}N$-AA with trophic transfer vs. the ammonia-excreting organisms examined in previous feeding studies (*e.g.,* zooplankton, bony fish, mollusks, etc.). We therefore propose that for marine mammals a multi-TEF calculation is required to

account for both ammonia and urea excreting 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 to study of mammalian, avian, (and also general terrestrial) ecology and trophic structure. They suggest that currently assumed compound-specific isotope analysis TEF values may not be accurate for many animal groups. We propose that for marine mammals the simple dual-TEF approach we outline should be accurate, due to predominance of ammonia excretion in most marine animals, however for carnivores or omnivores in terrestrial systems more complex relationships may be common.

INTRODUCTION

Stable isotope analysis has been widely used to understand the diet, migration, and food web dynamics of marine organisms, 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, red blood cells, etc.; 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 to tissue synthesis, and variable trophic enrichment factors (Martinez del Rio et al. 2009, Newsome et al. 2010). In the last two decades application of a new approach, compound-specific isotope analysis, has grown rapidly in isotope ecology, allowing access to more information about an organism's physiology and biochemistry (Hare et al. 1991, Fogel et al. 1997, McClelland and Montoya 2002, Fogel & Tuross 2003, Corr et al. 2005, Popp et al. 2007, Martinez del Rio et al. 2009). Isotopic analysis of individual organic compounds has now 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, Martínez del Rio et al. 2009, Popp et al. 2007, Larsen et al. 2011, McMahon et al. 2010). 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 and 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 for the first time 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), while simultaneously offering a new tool for far more precise estimates of trophic position (TP) in organisms (Chikaraishi et al. 2009).

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 and Montoya 2002, Chikaraishi et al. 2009).

A critical underlying issue for using compound-specific isotopes of AA to interpret trophic structure of wild populations is therefore to understand the magnitude of ^{15}N-enrichment with each trophic step. Prior workers have 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). Studies to date have focused primarily on the TEF for glutamic acid (Glu) and phenylalanine (Phe), representing the most consistent of the trophic and source AA groups ($TEF = \Delta_{Glu} - \Delta_{Phe}$; *e.g.,* McClelland and 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 $\delta^{15}N$ 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 ($\Delta_{Phe} \sim 0$ to 0.4 ‰), whereas the Glu typically shows greatest enrichment ($\Delta_{Glu} \sim 7.0$ 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 a wide variety of 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 (Styring et al. 2009, Naito et al. 2010). Calculated TP results from field studies have mostly been consistent with the assumption of widely similar TEF values.

However, recent data from some wild top marine carnivores have also strongly suggested that the core assumption of constant TEF values may need to be

revaluated for some animal groups (Lorrain et al. 2009, Dale et al. 2011). Despite the enormous potential of compound-specific N isotopes analysis for ecological studies of elusive, difficult to observe carnivores (such as marine mammals), there are no published feeding studies that have specifically examined how $\delta^{15}N$ 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 current application of compound-specific amino acid TP studies for a marine mammal. Specifically, to determine whether the overall $\delta^{15}N$-AA patterns from a marine mammal are comparable to those determined previously for lower TP organisms, and to examine if the widely used TEF values originally derived from plankton are in fact applicable for marine mammals 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 (< one year in age; Table 1; n=7), directly before release to the wild or medically-required euthanasia. Time at TMMC ranged from 1 to 93 days, however, only seals fully equilibrated to diet (> 14 days, n=6) were

used for TEF and TP calculations (Germain et al. 2011). Blood samples were collected in no-additive collection tubes from the epidural vein. The blood was centrifuged, and the serum was separated and then stored at -80°C. Pectoral muscle tissue was collected in some post-mortem individuals and stored at -80°C (Supplementary Table 1, n=8).

Seals at TMMC were fed exclusively herring (*Clupea harengus*). 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. All herring were wild caught in the same local fishery, and stored frozen in a large batch to provide food for all pinnipeds. We expect that all herring batches would have similar δ^{15}N-AA values, as they are sourced from similar ocean regions and time frames. Nevertheless, to account for any possible variation in δ^{15}N values from different herring batches, fish were collected from multiple batches throughout this experimental period, combined, and ground to a homogenous mixture for our compound-specific isotope analysis.

To further verify the direct trophic linkage between the composite herring food sample and analyzed harbor seals, δ^{13}C-AA values were also measured in both seal serum and the homogenized herring samples. The δ^{13}C values of essential AAs (having carbon skeletons the animals can not synthesize) have been shown to be unchanged with trophic transfer, and to therefore correlate strongly with the δ^{13}C value of the same AA in diet when diets are equilibrated (O'Brien et

al. 2005, McMahon et al. 2010). Linear regressions of $\delta^{13}C$ values of the essential AAs (Supplementary Fig. 1) for seals vs. the herring food mixture shows a high correlation ($R^2 = 0.94$), confirming the expected direct linkage between the AAs in our sampled seals and their composite herring food sample. This result also indicates that $\delta^{13}C$ values for essential AAs were fully equilibrated to the herring diet in harbor seal serum. Consequently, we infer that $\delta^{15}N$-AA values have also equilibrated to diet, consistent with previous bulk $\delta^{15}N$ data (Germain et al. 2011). A full discussion of $\delta^{13}C$ values and methods is beyond the scope of this paper, however additional methods detail is supplied in Supplementary Material.

Bulk and compound-specific amino acid isotope analyses.

Measurement of bulk $\delta^{13}C$ and $\delta^{15}N$ values on these samples is described in Germain et al. (2011). 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, 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 (per mil, ‰) 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 6N HCl acid hydrolysis and the formation TFA ester derivatives, following published McCarthy lab protocols (*e.g.,* McCarthy 2007, Sherwood et al. 2011; more detailed compound-specific isotope AA analytical descriptions are also provided in supplementary material). We determined $\delta^{15}N$ values for 14 AAs, each measured four 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 to be consistent other compound-specific isotope studies.

TEF, TP estimates, and statistical tests. We define the TEF ("Trophic Enrichment Factor", as introduced above) for Glu and Phe, which are generally accepted as best trophic and source AAs for TP estimates, as follows:

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

Alternate TP estimates based only on the bulk $\delta^{15}N$ values were also determined,

as described in Germain et al. (2011):

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

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 two trophic steps.

Compound-specific isotopes analysis of AA estimated TP using calculated differences between Glu and Phe in seal serum (or muscle) samples, using either the currently widely single-TEF approach (after McClelland and Montoya 2002) or a new multi-TEF equation proposed here. For the single TEF approach, we used the equation of Chikaraishi et al. (2009):

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

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 $TEF_{Glu\text{-}Phe} = 7.6‰$ for ammonia-excreting organisms.

For the multiple-TEF equation (as described in the discussion, and also supplementary material), accounts for both calculated TEF's in urea-excreting and ammonia-excreting organisms:

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

where TEF_{Urea} is the $TEF_{Glu\text{-}Phe}$ value for seals and other urea excreting organisms, and $TEF_{Ammonia}$ is the $TEF_{Glu\text{-}Phe}$ value for ammonia excreting organisms

(Chikaraishi et al. 2009). A more detailed derivation of the equation (4) is presented in the supplementary material.

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). Significant results are reported when P-values were less than 0.05. Statistical analyses were preformed in JMP (ver.7) and Matlab.

RESULTS AND DISCUSSION

Overall δ^{15}N-AA patterns: δ^{15}N values of individual amino acids.

Figure 1 and Table 1 present the first patterns for δ^{15}N-AA values reported for a living pinniped. The δ^{15}N values for seven 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 (discussed in more detail below). The pattern in δ^{15}N-AA values for all seals generally corresponds well with expectations from prior studies. The source AAs (Gly, Lys, Ser, Tyr, Phe, and Arg) all have lower δ^{15}N values, presumably reflecting more closely the δ^{15}N values of AAs in autotrophs at the base of the food web (Popp et al. 2007), whereas the trophic AA (Ala, Asp, Glu, Ile, Leu, Pro, and Val) all have significantly elevated δ^{15}N values, linked to ^{15}N enrichment from deamination/transamination associated with trophic transfer (McClelland & Montoya 2002, Macko et al. 1986). The pattern in seals can also be compared

more widely with previous data for diverse heterotrophic organisms (Fig. 2); all data sets have similar patterns that holds 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 Pro was consistently the most ^{15}N-enriched AA in every harbor seal. In prior studies on plankton and fish, Glu is typically observed as the most enriched AA (*e.g.,* McClelland and 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 et al. 2010). However, several recent studies on higher TP animals also have also reported Pro to be the most ^{15}N-enriched AA (*e.g.*, penguins and ancient humans; Styring et al. 2009, Lorrain et al. 2009), and it has in fact 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.,* McClelland & Montoya 2002, Chikaraishi et al. 2009, Macko et al., 1987), the elevated Pro δ^{15}N in our seals and recent literature data likely indicates that Pro has variable enrichment with trophic transfer (*i.e.,* that $\Delta_{Pro} > \Delta_{Glu}$ in selected higher TP organisms). An additional possible factor might be the central role of

Pro in the formation of collagen. Collagen is important in skin, bone, and connective tissues (making up ~20% of the body mass of humans, and nearly 30% of protein mass; Di Lullo et al. 2002) and every third AA in collagen is either Pro or hydroxyl-Pro. Linkage of elevated Pro $\delta^{15}N$ 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 $\delta^{15}N$ 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 a poor choice for determining TP.

Thr has notably divergent $\delta^{15}N$ values vs. other AAs (Fig. 1). The extreme Thr ^{15}N-depletion observed in seals is consistent with some past heterotroph data (Fig. 2; Hare et al. 1991, Popp et al. 2007, Sherwood et al. 2011), but 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 some early work, but instead that Thr becomes progressively depleted not only with increasing TP, and also to a far greater extent in at least some long-lived organisms. For these reasons, we have designated it a "metabolic" AA in our figures (MB). Factors proposed to date for low Thr values include successive trophic transfers (Styring et al. 2009), and nutritional stress (Hare et al. 1991), both 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. 2). We note that Thr (like Pro) is biochemically also involved in the formation of collagen and elastin (Bowes & Kenten 1949), suggesting that lower values in marine mammals could also be linked blubber formation. While our data cannot differentiate between specific mechanisms, they do suggest 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.

Figure 3 compares Δ values from a recent compilation of trophic transfer data for δ^{15}N-AA values (Chikaraishi et al. 2009), with those found in harbor seals in this study. As note above, the Δ values are the difference of an individual AAs between the consumer and food, with a single trophic transfer. We observed large differences in Δ values vs. those determined in lower TP marine organisms. Because of differences in the exact AAs measured by different analytical approaches, it is not possible to directly compare Δ values for all AA, however for the majority of AA 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 have would be expected to be relatively similar (*e.g.,* McClelland and Montoya, 2002). Because we also have the most directly comparable AA data for

trophic AA, the differences seem particularly clear: five of seven trophic AA 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).

The Δ values for Phe, however, remain close to 0‰ in both data sets. This further strengthens the argument that Phe is the "best" (and perhaps only) functional source AA (*i.e*, undergoing almost no $\delta^{15}N$ change with trophic transfer), and therefore represents the most accurate AA proxy for $\delta^{15}N$ 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 and Montoya 2002, Chikaraishi et al. 2009). The Δ value for Glu in harbor seals is much lower than the previous value (Δ = 2.9 ± 0.6‰), quite similar to the Δ values for other main trophic AAs (Ala, Asp, and Leu). Together, these observations suggest that the nitrogen metabolism of many AA with trophic transfer, and particularly those in the trophic group, may be quite different in harbor seals (and likely other marine mammals), than in the invertebrates and fish used to determine Δ and $TEF_{Glu\text{-}Phe}$ values used to date.

The lower Glu Δ values, coupled with stable Phe $\delta^{15}N$, 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‰) found in zooplankton, gastropods, and fish (McClelland and Montoya 2002, Chikaraishi et al. 2007). An unpublished PhD dissertation by Zhao also previously measured compound-specific isotopes of AA of one adult harbor seal and its food, using an alternate off-line analytical approach (Zhao 2002). 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 very similar to our data for the same species. Further, recent literature has also indicated low $TEF_{Glu\text{-}Phe}$ values for both wild birds and skates (Fig. 4). Lorrain et al. (2009) used a linear-mixed effects model to estimate $TEF_{Glu\text{-}Phe}$ for penguin blood and obtained a value of 3.7 ± 0.2‰, again statistically indistinguishable from the value for harbor seal data sets (Fig. 4). Wild stingrays 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 $\delta^{15}N$-AA values. In contrast, data from some bony 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 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 narrow but distinct ranges (Fig. 4).

Is variable $TEF_{Glue\text{-}Phe}$ tied to trophic position and/or urea vs. ammonia excretion? There are a number of possible explanations for the Δ and $TEF_{Glu\text{-}Phe}$ differences we observe 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 $\delta^{15}N$ 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 broad division evident in Fig. 4 does not support this as a primary explanation; in particular the similar $TEF_{Glu\text{-}Phe}$ values derived from growing pups (this study) and one adult harbor seal (Zhao 2002) suggests animal age is not a major factor.

Elevated TP itself might also contribute to differences in Δ values, because of differences in the both quality and quantity of diet, in particular of dietary protein (Robbins et al. 2010, Florirn et al. 2011). Animals with a higher TP consume diet rich in protein (*i.e.,* high quantity) likely have AA compositions closer to that of their own body tissues (*i.e.,* high quality). Theory and experiment both suggest that protein quantity quality may alter bulk $\delta^{15}N$ Δ values (*e.g.,* Sick

et al. 1997). However, the seals in this study were in fact not particularly high in TP vs. either 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 the assumed $TEF_{Glu\text{-}Phe}$ values (~ 7.6‰), when applied to high TP wild fish (tuna; TP 4 to 5), returned reasonable TP estimates from δ^{15}N-AA data. Further, the recent feeding studies noted above also showed higher $TEF_{Glu\text{-}Phe}$ values for other fish at nearly the same TP (Chikaraishi et al. 2009, Bloomfield et al. 2011). Together, while some linkage between elevated TP and $TEF_{Glu\text{-}Phe}$ cannot be ruled out, this seems very unlikely to be the primary factor for the low $TEF_{Glu\text{-}Phe}$ values for the single trophic transfer examined in these seals.

In contrast, a linkage of Δ and $TEF_{Glu\text{-}Phe}$ values with metabolic differences associated with ammonia vs. urea production would be consistent with all observations. Most marine organisms, including all those on which the traditional $TEF_{Glu\text{-}Phe}$ values have been determined, produce ammonia (McClelland and 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 would therefore provide a unifying framework for $TEF_{Glu\text{-}Phe}$ offsets of the disparate organisms in Figure. 4. 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). 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 $\delta^{15}N$ values remains 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 our hypothesis is plausible (Fig. 5). The Glu pool is the precursor for both ammonia and urea waste (*e.g.*, Champe et al. 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 (*e.g.,* Checkey, 1989), and therefore strongly ^{15}N enriched Glu, and corresponding large $TEF_{Glu\text{-}Phe}$ values. In contrast, urea has two nitrogen groups and two 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 et al. 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. We also note that a TEF difference in bulk $\delta^{15}N$ values related to urea vs. ammonia-excretion has been suggested in at least one meta-analysis based on bulk isotopic data (Vanderklift and Posnard, 2003). However, in that study the difference in bulk $\delta^{15}N$ per trophic transfer for urea excretion was higher than ammonia excretion, in contrast to what might be expected from the compound-specific isotopic AA results. While this further points to differences in TEF values based on N excretion products, it also highlights the need for additional, more focused research in this area. Overall, while we do not have data here to test any specific mechanistic explanation, the fundamental differences in urea vs. ammonia N pathways seem consistent with the overall hypothesis of characteristic different $TEF_{Glu\text{-}Phe}$ value ranges.

Implications for compound-specific isotopes of AA based trophic studies: a multi-$TEF_{Glu\text{-}Phe}$ equation for urea-excreting organisms. Several closely related calculations have been used to estimate TP of organisms using $\delta^{15}N$-AA values (McClelland and Montoya 2002, McCarthy et al. 2007, Popp et al. 2007), differing primarily in the specific trophic and source AAs chosen in the equation. Some researchers have also included multiple trophic and source AAs in order 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 and Montoya 2002, Chikaraishi et al. 2009, Popp et al. 2007, Hannides et al. 2009). If $TEF_{Glu\text{-}Phe}$ values for urea-excreting organisms are in fact much lower, this could have a significant impact on conclusions of any study which includes mammals and birds.

The likely validity of our lower $TEF_{Glu\text{-}Phe}$ estimates can be evaluated in several ways. First, we compared results using the currently assumed $TEF_{Glu\text{-}Phe}$ 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 on wild-caught herring essentially rules out this result: our seals could have a TP of ~2 only if the wild herring were strictly herbivores (had TP =1). While young herring do feed primarily on plankton, adults have a more diverse diet, comprising zooplankton, small fish and fish larvae (*e.g.,* Foy and 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 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 also scat analysis also predict the seals to be feeding at higher

TP (Table 2; Germain et al. 2011). 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 other marine mammals), independent $TEF_{Glu\text{-}Phe}$ values for urea vs. ammonia excretion must be explicitly taken into account. This approach is based on the assumption that isotopic shifts can be modeled in two 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 essentially known to be the case in our study, since seals are fed only herring), such that a $TEF_{Ammonia}$ of 7.6‰ can be applied. The second stage represents only the final trophic transfer from ammonia-excreting prey to urea-excreting seals, for which a TEF_{Urea} of 4.3‰ is applied. Using the assumption that TEF_{Urea} applies to only one trophic transfer, a multi-$TEF_{Glu\text{-}Phe}$ trophic transfer estimate can then be derived using only measured $\delta^{15}N$ values for both Glu and Phe in seals (complete derivatization provided in supplementary material):

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

Using equation (4), 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, than average seal TP value (2.2) would be far too low for these marine carnivores. Alternately, if we instead 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 might be possible for seals, but it also would require that the lower $TEF_{Glu\text{-}Phe}$ value is in fact typical of *both* herring and all their 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, incorporating distinct $TEF_{Glu\text{-}Phe}$ values both ammonia and urea excretion, can provide an accurate TP estimates at least in seals.

CONCLUSIONS

This first compound-specific isotopic analysis of AA data from a controlled feeding study of a marine mammal have 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 previously expected δ^{15}N-AA patterns, suggesting unique aspects to biochemical pathways for these AA. Major differences were also observed in Δ and TEF values for many AA relative to expected values. In particular, the $TEF_{Glu\text{-}Phe}$ value 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

($TEF_{Glu\text{-}Phe}$ ~ 7.6‰). We hypothesize that this is most likely linked to the N-AA metabolic differences associated with ammonia vs. urea production as major N waste products. Specifically, our results suggest that the biochemical pathways used to synthesize ammonia and urea waste are linked to different patterns of $\delta^{15}N$ fractionation of AA with trophic transfer, which must be accounted for when using $\delta^{15}N$-AA to estimate TP. While compound-specific isotope AA data from urea excreting animals is not yet extensive, comparison to existing literature data strongly supports this conclusion.

Taken together the existing data suggest that distinct, but relatively narrow, ranges of $TEF_{Glu\text{-}Phe}$ exist for ammonia vs. urea excreting animals. We therefore propose that a multi-$TEF_{Glu\text{-}Phe}$ calculation can be used (and is likely required) to accurately estimate TP in marine mammals. Using a multi-$TEF_{Glu\text{-}Phe}$ approach for these 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. While we cannot fully rule out some change in TEF values based on increasing TP, this alone could not explain our main results. However, we also note that these two mechanisms are also not mutually exclusive, and more extensive experiments on high TP organisms will likely be required. 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 urea excreting animals. For most marine mammals this assumption is reasonable, since the vast majority

of organisms in marine food webs (and therefore most important prey marine mammal prey species such as squid, fish, and shellfish) 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 implication 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 specimens by ~0.5 TP (Styring et al. 2009, Naito et al. 2010), consistent with implications of this study. For terrestrial systems in which carnivores or omnivores may have diets which include significant urea excreting prey, our data further suggest that a more complex multi-TEF approach would be required than that presented here, and that δ^{15}N-AA data might ultimately need to be constrained by independent dietary information. We suggest that work examining TEF values in urea excreting animals, as well as higher TP animals, 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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Name	Sample #	Fraction	Sex	Age	R.D.E	Days in Center	Health	Death
Nigel	1696	Serum	M	P	R	*62*	-	Released
Hang Ten	1698	Serum	F	P	R	*83*	-	Released
BMB	1704	Serum	F	P	D	*36*	3	Colitis
Luka	1705	Serum	F	P	R	*93*	-	Released
Nabby	1718	Serum	F	P	E	*62*	6	Neurological
Stumpy May	1739	Serum	F	P	E	*18*	3	Aspiration; Enteritis
Shenanigans	1748	Serum	M	P	E	1	3	Spine Trauma
Fish Feed	Fish Feed	Whole						

Ala	Gly	Thr	Ser	Val	Leu	Ile	Pro	Asp	Glu	Phe	Lys	Tyr	Arg	Bulk
27.2	12.9	-17.0	9.1	23.5	24.0	15.5	28.1	18.1	23.7	9.5	14.0	20.3	17.7	16.4
24.1	10.3	-25.4	9.7	17.7	23.7	19.4	29.4	18.7	23.4	10.2	13.5	19.0	18.6	16.5
22.1	12.7	-22.8	10.8	19.7	23.2	22.7	28.7	17.5	22.7	10.7	5.6	19.3	-	15.8
23.3	9.6	-27.5	9.1	17.1	23.3	21.5	28.3	18.2	23.3	9.6	11.5	17.3	15.8	16.3
21.9	10.2	-18.6	9.6	22.1	22.8	18.2	26.8	18.8	24.4	9.1	7.0	19.6	-	15.4
20.1	15.2	-7.6	13.0	16.7	17.1	1.8	21.4	16.2	19.7	11.9	3.5	20.7	9.2	12.4
26.8	18.9	-21.4	19.8	26.6	25.8	20.8	29.0	21.4	25.8	12.7	16.7	14.1	-	18.0
21.9	4.3	-13.6	1.7	13.8	21.7	21.2	22.7	15.7	20.6	11.3	8.6	14.1	-	12.5

Table 1. Amino acid $\delta^{15}N$ of captive, rehabilitating harbor seals recovering at The Marine Mammal Center in the Spring of 2007.
All AA measurements were repeated 4 times with a average SD of 0.9 ± 0.2‰. Sex: Male (M), Female (F); Age: Pup (P); R.D.E.: Released (R,), Death (D), Euthanized (E). Health status on a scale of 1-7, where 1 is starving and 7 is obese nutritional state according to blubber thickness. Data and TEF calculations within the text is extrapolated from HS serum samples for seals in TMMC greater than two weeks (*i.e.,* equilibrated diet) compared to ground-up fish feed.

Calculation Method	Bulk	Scat	Single-TEF*	Single-TEF	Multi-TEF
TEF Values	N/A	N/A	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	-

Table 2. Results of different trophic position calculations for captive harbor seals and their herring food.
Estimated average trophic positions (TP) and ranges derived from different methods. Bulk = estimated TP based from bulk serum isotopes and varying $\delta^{15}N$ sources (Germain et al. 2011). Scat TP estimate is from literature, based on past harbor seal scat analyses from similar CA region (Tollit et al. 1997). Single TEF* indicates use of equation (3) and a TEF value of 7.6 originally from McClelland and Montoya (2002); Single-TEF uses equation (3), but using our measured $TEF_{Glu\text{-}Phe}$ value of 4.3 for tested harbor seals. Multi-TEF uses equation (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 and Posnard 2003).

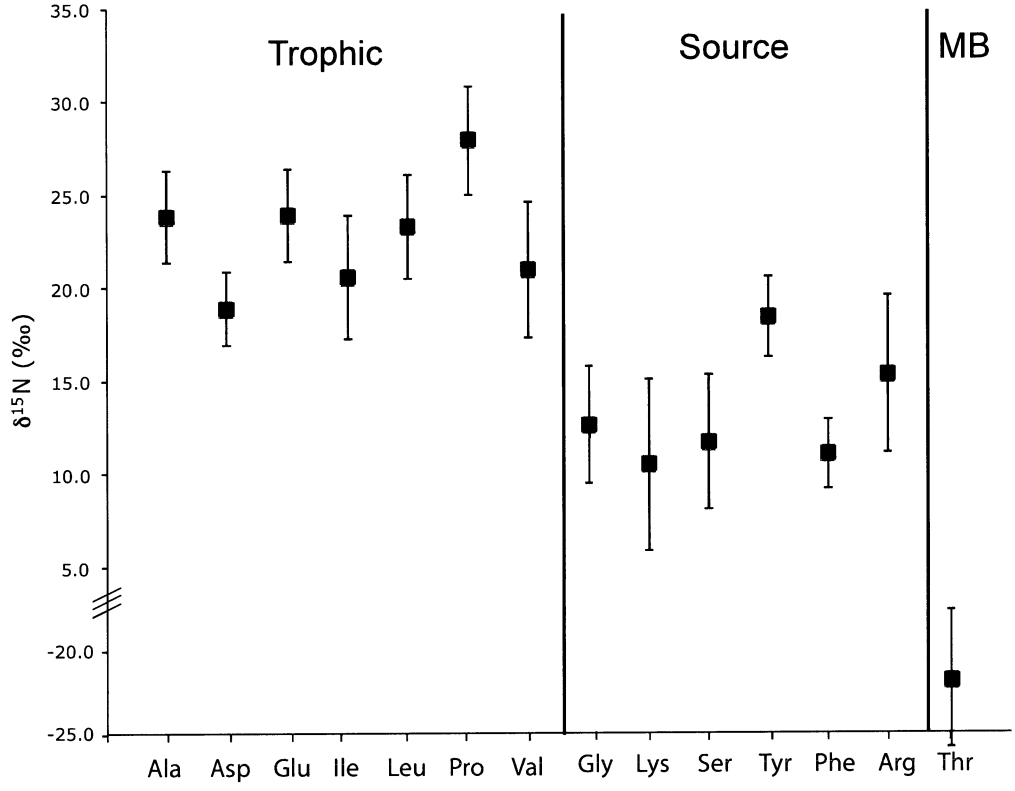

Figure 1. δ^{15}N-AA pattern for captive harbor seals (*Phoca vitulina*). Measured (*i.e.*, non-normalized) individual amino acid data are reported, arranged according to the trophic, source and metabolic (MB) amino acid groupings. Error bars indicate 1 standard deviation (n=7 seals); AA abbreviations are defined in *Methods*. Note scale break required for inclusion of extremely depleted Thr δ^{15}N value.

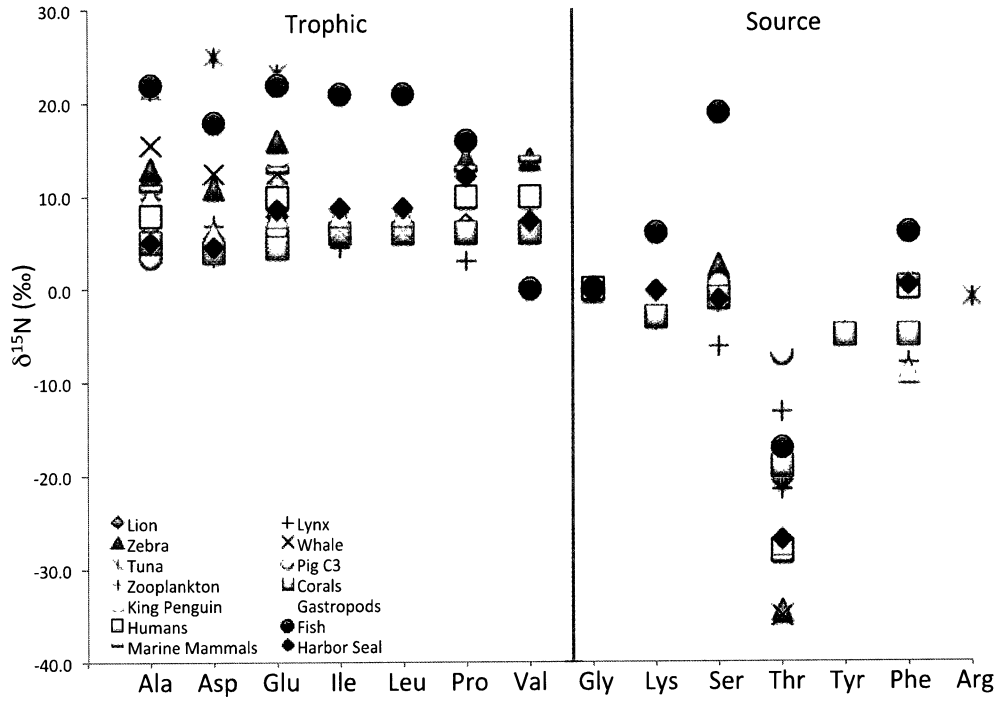

Figure 2. Comparison of δ^{15}N-AA patterns across animal species. Measured (normalized to Gly) δ^{15}N data for harbor seals (this study) vs. literature data on other animal species. Patterns of δ^{15}N values are similar across broadly phylogenetically distant taxa. Trophic AAs are always enriched in ^{15}N relative to the source AAs. Data sources: Hare et al. 1991 (lion, lynx, zebra, whale, pig C3), McClelland and Montoya 2002 (zooplankton), Popp et al. 2007 (tuna), Chikaraishi et al. 2007 (fish, gastropods) Styring et al. 2009 (humans, marine mammals), Lorrain et al. 2009 (king penguin), and Sherwood et al. 2011 (corals). Refer to legend for specific symbols. Amino acid abbreviations are as defined in text (*methods)*.

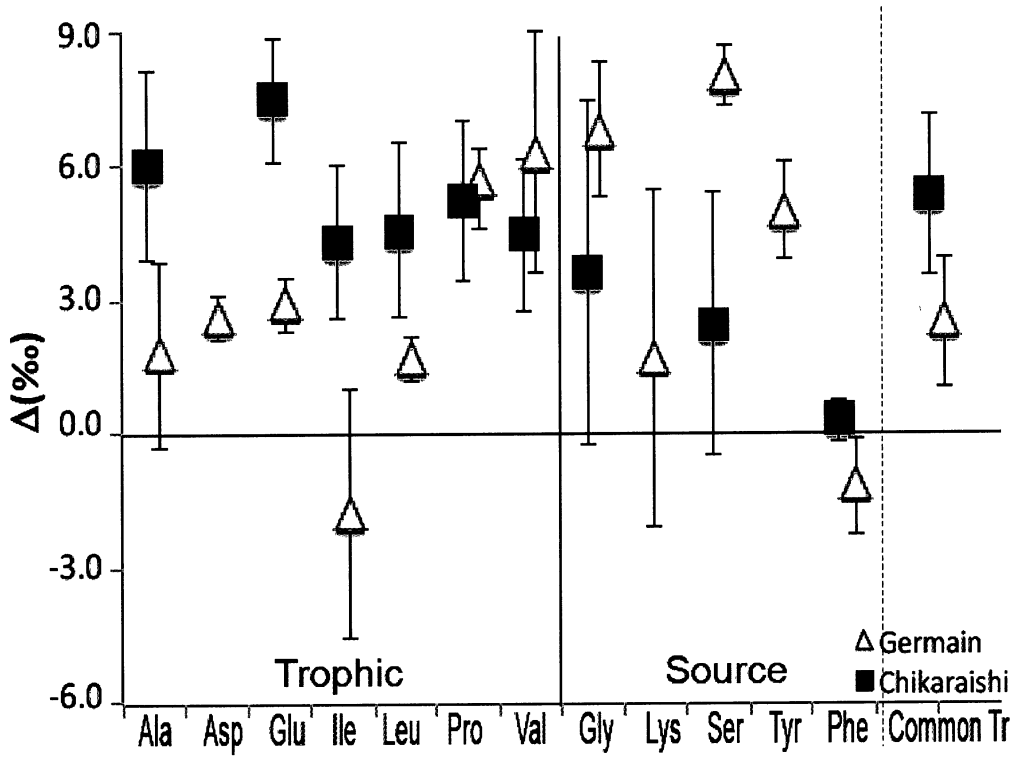

Figure 3. Trophic transfer effect on individual δ^{15}N-AA values for harbor seals vs. diverse ammonia-excreting marine organisms. $\Delta(\delta^{15}NAA_{consumer} - \delta^{15}NAA_{food})$ values for harbor seals in this study are indicated by open triangles; error bars are 1 standard deviation (n=6). Average $\Delta\delta^{15}N_{ConsumerAA\text{-}FoodAA}$ values determined for a range of other marine consumers (all reported to have similar overall values; Chikaraishi et al. 2009), and are indicated by black squares, with error bars representing 1 standard deviation 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 *methods* section. Common Tr = average of all trophic AA Δ values that were directly comparable between data sets.

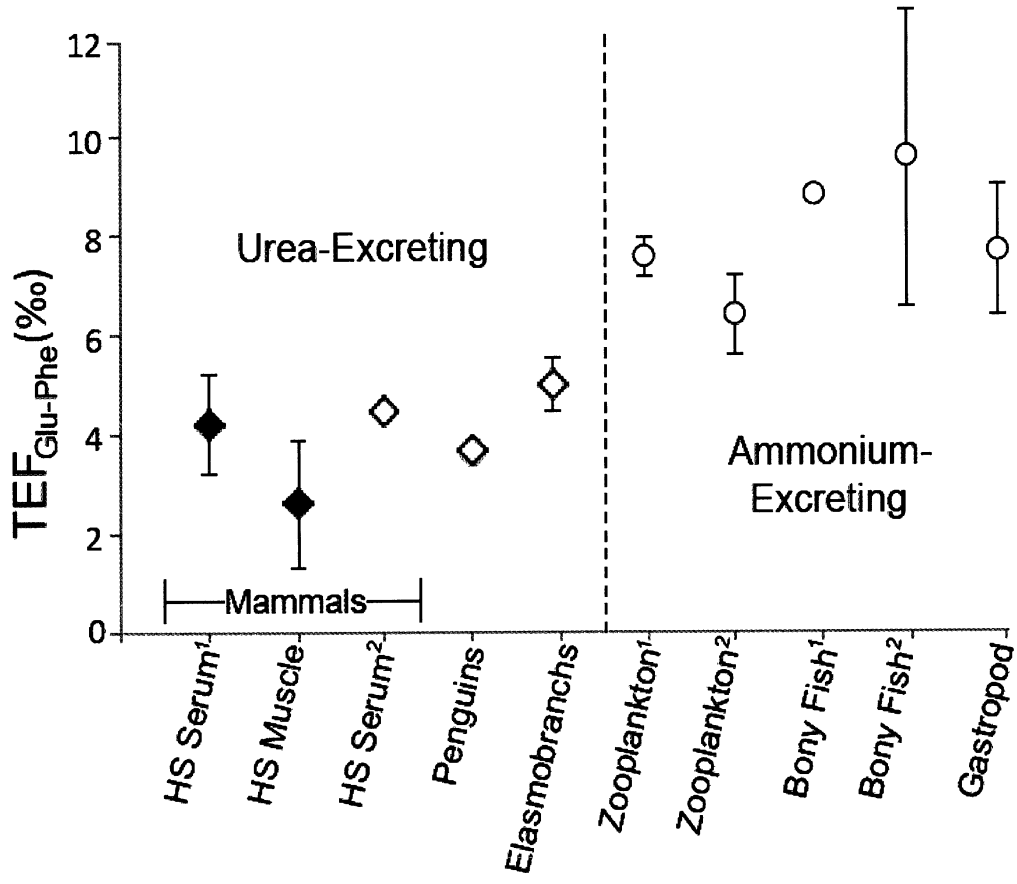

Figure 4. Comparison of trophic enrichment factors ($TEF_{Glu\text{-}Phe}$) data for ammonia-excreting vs. urea-excreting animals. A clear bifurcation in existing $TEF_{Glu\text{-}Phe}$ data suggests that distinct ranges exist for organisms linked to major nitrogen waste product. Filled diamonds are data from this study (harbor seal serum[1] and muscle). Open diamonds are past literature data for harbor seals[2] (Zhao dissertation), penguins (Lorrain et al. 2009), elasmobranchs (stingrays; Dale et al. 2011). Open circles for ammonia excreting organisms (zooplankton[1], bony fish[1], and gastropods, Chikaraishi et al. 2009; zooplankton[2], McClelland and Montoya 2002; bony fish[2], Bloomfield et al. 2011). Error bars indicate 1 standard deviation.

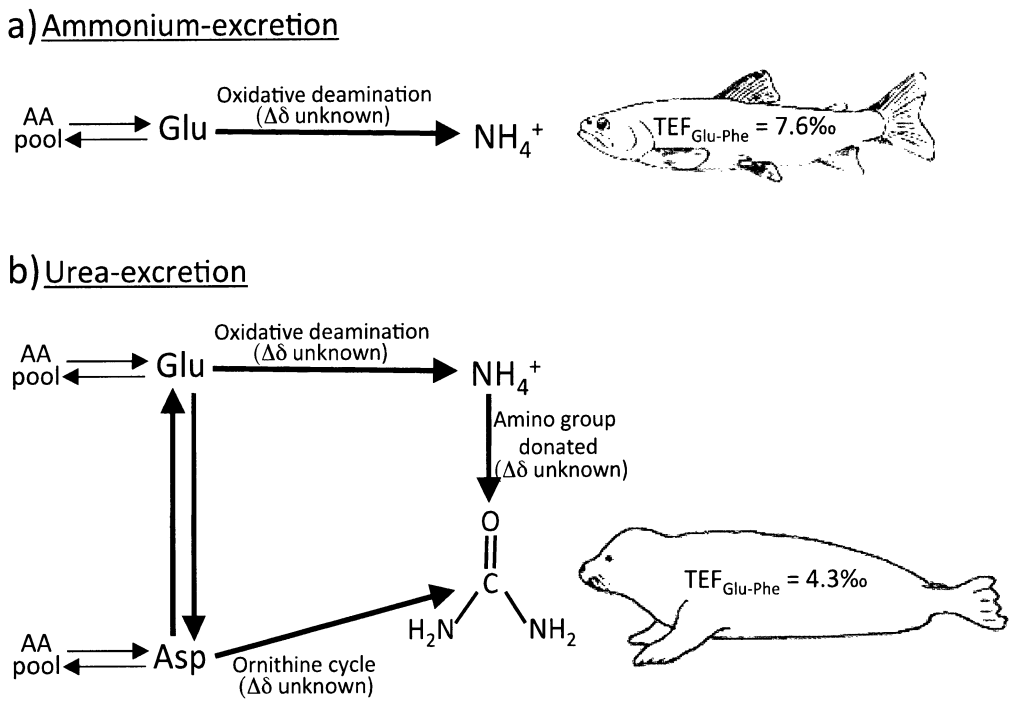

Figure 5. Biochemical pathways for N transfer from glutamic acid to ammonia and urea. Cartoon illustrates Glu pool as the precursor for N in both ammonia and urea. a) Ammonia is produced directly through oxidative deamination of glutamate, therefore with no fractionation. Animals shown to have higher $TEF_{Glu\text{-}Phe}$ values (~7‰) to date all excrete ammonia. b) Urea, in contrast, has two 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‰) area associated with animals using this pathway due to an equilibrium effect between Glu and Asp dependent upon reservoir times.

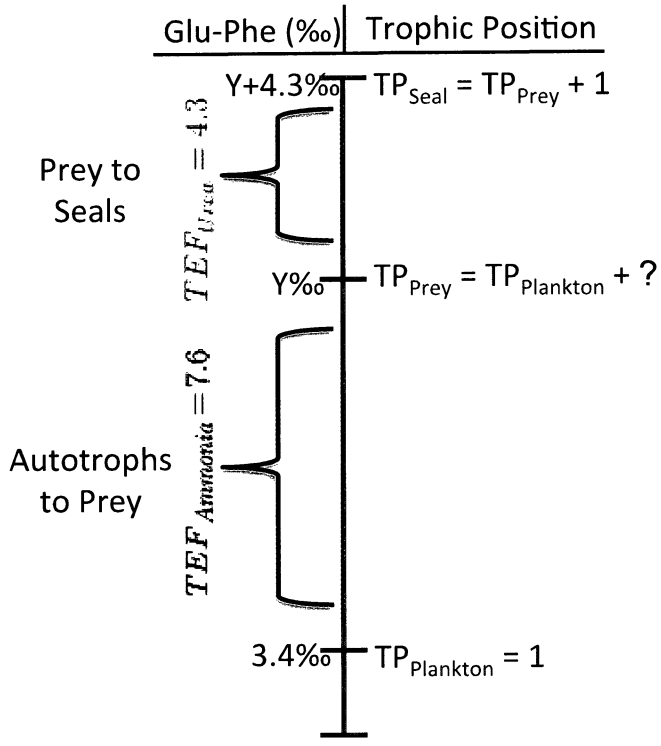

Figure 6. Cartoon illustrating multi-TEF tropic position (TP) approach for marine mammals. Key assumptions are that marine mammals feed uniquely on ammonia-excreting organisms, so that trophic transfers for all prey can be modeled with a single $TEF_{Ammonia}$ of 7.6‰. A single final trophic transfer from prey to seals is assumed, for which a TEF_{Urea} of 4.3‰ can be applied. At base of food web Glu-Phe value in autotrophs is assumed to be 3.4‰ (DeNiro and Epstein 1981, Minagawa and Wada 1984). Y‰ indicates measured 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 assumed to be Y+4.3‰, to account for an additional urea-excreting trophic transfer. All TEF references indicate $TEF_{Glu\text{-}Phe}$, with subscripts shortened for simplicity.

CHAPTER 3:

Carbon and nitrogen isotopes in amino acids reflect feeding behavior and nutritional status differences between populations of harbor seals in California

Germain, L.R., P. L. Koch, J. T. Harvey, and M.D. McCarthy

ABSTRACT

Harbor seals (*Phoca vitulina*) are the most widely distributed of pinniped species, commonly found in waters off the majority of coastline in the Northern Hemisphere. However, specific regional populations have been decreasing over recent decades possibly due to prey distribution changes. One of these locations is San Francisco Bay (SFB), a highly urbanized estuary, with a small population of a few hundred seals that forage in these waters year-round. To assess whether this group forages on lesser quality prey, we compared these seals to healthier, stable populations in Tomales Bay and the Channel Islands. We examined the $\delta^{13}C$ and $\delta^{15}N$ of fourteen individual amino acids (AA) of between young and older seals, using compound-specific isotope analysis, a growing tool in ecological studies, to broaden our understanding of foraging behavior (i.e., source of prey and trophic position). The $\delta^{13}C$-AA values are placed in three distinct groups, essential AAs which are directly derived from diet, conditionally essential AAs that can come from diet under certain physiological conditions, and non-essential AAs that are biosynthesized. The data suggested that older vs. younger seals in Channel Islands and Tomales Bay were consuming prey from different sources, whereas in SFB the different aged seals were feeding on similar prey, but were undergoing different physiology. The $\delta^{15}N$-AA values also suggested differences between age classes and location. In the Channel Islands, older seals were found to be feeding at a higher trophic position than the younger seals, whereas in SFB, the opposite was

found. The younger seals were estimated to be feeding at a higher trophic position, most likely an artifact from recently weaning their mother's milk for the weaners, and possibly an indicator of nutritional stress for yearlings. The combination of these analyses implies more detailed and accurate dietary information of an animals foraging ecology can be inferred using this new method, which can be invaluable tool in difficult to observe environments.

INTRODUCTION

Eastern Pacific harbor seals (*Phoca vitulina richardii*) occur in near-shore waters ranging as far south as Baja California up to the Aleutian Islands (Bigg 1981, Carretta et al. 2001). Since the Marine Mammal Protection Act was passed in 1972, many harbor seal populations have increased in size along the eastern Pacific Rim (Harvey et al. 1990). A major goal of the act was to attain an optimum sustainable population within the limits of a specific habitat. Although harbor seals appear to have reached this optimum sustainable level at many locations in California and Oregon, aerial survey data suggests their population has not fully recovered in San Francisco Bay, California (SFB) (Hanan 1996, Grigg et al 2004). In SFB there is currently a population of ~500 individuals who spend year-round foraging, breeding, and resting in these highly urbanized waters, and thus represent a marine mammal population whose activities are completely impacted by conditions in the bay. There are multiple hypotheses as to why this population has not increased like those on the open coast, including anthropogenic disturbances (Kopec and Harvey 1995, Green and Grigg 2006), contaminants leading to lower reproduction rates through immunosuppression and disease (Neale et al. 2005), and decreased survival rates due to reduced prey availability or quality (Allen 1991, Grigg et al. 2004).

In order to examine hypotheses about trophic and nutritional status, past researchers have analyzed SFB harbor seal diet using scat analysis (Torok 1994,

Gibble 2011); they have also used satellite- and radio-transmitters to study foraging patterns and behavior (Nickel 2003). While all of these techniques are non-invasive, each has inherent limitations. Scat analysis relies exclusively on the identification of prey hard parts, which tends to be inaccurate for quantifying abundance of some soft-bodied prey, and small fish (Bowen 2000, Phillips and Harvey 2009). For example, in captive studies of harbor seals, fish under 30 cm, otoliths were underrepresented and were consistently degraded after digestion (da Silva and Neilson 1985, Harvey 1989). Likewise, scat analysis and tracking studies only represent a relatively brief temporal window. Scat analysis reflects the most recent diet of the animal at the time of sampling. Tagging/transmission studies do not directly reflect diet at all, but rather record foraging zone, with diet inferred based on assumptions about preferred prey and above prey abundance derived from surveys in given locations. Finally, stable isotope analysis of bulk tissues (e.g., muscle, bone collagen, whisker keratin, blood, etc.) has also been used to study foraging behavior, trophic dynamics, and migratory patterns in pinniped species (e.g., Burns et al. 1998, Hirons et al. 2001, reviewed in Newsome et al. 2010). Depending on tissues sampled, stable isotope analysis can integrate information about food sources, trophic position, and nutritional status over months to years. However, conclusions from bulk stable isotope analyses are often confounded by the use of multiple assumptions, such as source isotope values at the base of complex food webs, which can be highly variable.

Recently, compound-specific isotope (CSI) analysis of individual amino acids (AA) has emerged as a powerful new tool in ecology. Applications of this approach have grown rapidly in recent years, due to their ability to greatly refine the information derived from isotopic measurements about the dietary behavior, trophic dynamics, and nutritional status (e.g., Martinez del Rio et al. 2009, Newsome et al. 2010 and references therein). Early work revealed that specific fractionation patterns for ^{13}C-to-^{12}C and ^{15}N-to-^{14}N for autotrophic organisms are directly related to central metabolic biochemical pathways (Abelson & Hoering 1961, Macko et al. 1987). The pattern of isotopic values in the AAs in a heterotroph (or detrital material) therefore reflects the original autotrophic biochemical signature, subsequent trophic transfers, and potentially the degree of microbial degradation (McCarthy et al. 2004, 2007). However, recent research has underscored that in heterotrophic food webs, changes in AA carbon isotope ($\delta^{13}C$) values (reflecting the recycling vs. *de novo* synthesis of the AA carbon skeleton) are largely *decoupled* from changes in AA nitrogen isotope ($\delta^{15}N$) values (predominantly reflecting degree of transamination of the amine nitrogen group). Therefore, both $\delta^{13}C$-AA and $\delta^{15}N$-AA patterns carry unique, but complimentary, ecological information.

For $\delta^{13}C$-AA patterns, a key division is between the essential vs. non-essential AAs (termed "indispensable" vs. "dispensable" in some literature). Truly essential AAs must be derived only from diet, and therefore the $\delta^{13}C$ values of

these AAs remain unchanged and directly record $\delta^{13}C$ values in primary production at the base of food webs. The $\delta^{13}C$ values of essential AAs can also be diagnostic for different primary producers and bacteria (Larsen et al. 2010), and are unchanged up food chains (O'Brien et al. 2005, McMahon et al. 2010), such that $\delta^{13}C$ of essential AA can be used to indicate an animal's prey preferences (C3 vs. C4, or marine vs. terrestrial). In contrast, $\delta^{13}C$ values of the non-essential AA (and some termed "conditionally" essential AAs, like proline) can be used to explore isotopic routing vs. *de novo* synthesis (Howland et al. 2003, Jim et al. 2006). Non-essential AAs typically undergo relatively large fractionations with trophic transfer and their specific degree of fractionation has also been shown to be indicative of diet composition (i.e., protein vs. carbohydrate content; McMahon et al. 2010), growth rate, and metabolic state (Newsome et al. 2011).

The $\delta^{15}N$ values of AAs indicate isotopic values of primary production at the base of food webs, but also have a unique ability to directly indicate trophic position. Study of change in $\delta^{15}N$-AA values with trophic transfer have also revealed that AAs fall into two groupings, now commonly called "trophic" AAs and "source" AAs (Popp et al. 2007). The trophic AAs are greatly enriched in ^{15}N with each trophic transfer, while the source $\delta^{15}N$-AA values remain relatively unchanged, more closely reflecting isotopic values at the base of the food web (Hare et al. 1991, McClelland and Montoya 2002, Chikaraishi et al. 2009). Previous studies using CSI analysis have shown that the degree of enrichment

between specific trophic and source AAs (trophic enrichment factors, TEF) are highly predictable, providing the basis for a new molecular-level tool for evaluating trophic position far more precisely than is possible from bulk isotope values (McClelland and Montoya 2002, McCarthy et al. 2007, Chikaraishi et al. 2009, Germain et al., in review). Together, these factors create the unique potential to deconvolute variations in $\delta^{15}N$ values at the base of food web from the effect of subsequent trophic transfers, and have led to rapidly expanding research applying $\delta^{15}N$-AA values in diverse environments (Popp et al. 2007, Lorrain et al. 2009, Styring et al. 2009, Naito et al. 2010, Dale et al. 2011). However, at the same time, recent research has proposed that specific TEF values may also vary depending upon an animal's main form of nitrogen waste (e.g., urea vs. ammonia; Germain et al. in review), such that a specific new calculation approach is required for marine mammals in order to derive accurate estimates of trophic position from CSI analysis.

This study aims to apply coupled δ13C-AA and δ15N-AA analysis for the first time in a marine mammal to assess foraging behavior and nutritional status of harbor seals in highly urbanized SFB vs. those in healthy, stable populations on the open California coast. We will assess relative $\delta^{13}C$-AA and $\delta^{15}N$-AA patterns as indicators of dietary information, such as types of prey consumed, trophic position, and metabolic activity of individual seals, comparing CSI-AA information to inferences drawn from bulk isotope values and other more

traditional approaches (e.g., scat analysis). For δ^{15}N-AA data, we will also be able to broadly compare trophic position predictions using different CSI-AA approaches, as well as bulk δ^{15}N values. While a few earlier studies have examined CSI-AA data from captive animals in controlled environments, to our knowledge this is the first CSI-AA data reported for any wild marine mammal population, and in fact one of the first with coupled δ^{13}C-AA and δ^{15}N-AA data for living animals (Lorrain et al. 2009 is another). Finally, by comparing our AA results for seal populations from different locations with minimal anthropogenic disturbances vs. SFB, we will examine if CSI-AA data and trophic factors can indicate nutritional stress in specific individuals.

MATERIALS AND METHODS

Study sites and harbor seal sampling. Wild harbor seal serum and red blood cell fractions were collected from northern and southern California during May - June 2007 (NMFS Research Permit no. 555-1565). Blood was drawn from the epidural veinous sinus into non-additive red-top collection tubes, allowed to clot, centrifuged, and separated into serum and red blood cell fractions. The fractions were then frozen and stored at -80°C, followed by lyophilization, and homogenization. Lipids were removed following the protocol of Dobush et al. (1985) in a Dionex Accelerated Solvent Extractor, where samples were rinsed

twice with 100% petroleum ether at 50°C and 1500 psi, held in 60% volume for 5 min, and finally dried under a fume hood to remove residual solvent. Samples were then stored in a desiccator for further chemical and isotopic analysis. The UCSC Institutional Animal Care and Use Committee Protocols approved all animal sampling procedures.

Males and females were assigned to the following age classes based on their weight and length measurements (Bigg et al. 1969): adult (A), > 4 yr; subadult (SA), 2 – 4 yr; yearling (Y), 1 – 2 yr; and weaner (W), 1 mo – 1 yr. Tomales Bay (TB; 38° 13.9'N, 122° 58.1'W), located approximately 65 km north of SFB, is 19 km long and relatively shallow (Fig. 1). Seals were sampled near the entrance to the Bay. TB is adjacent to Point Reyes National Seashore, and is a relatively undisturbed environment supporting an array of invertebrates, fish, seals and seabirds. The seals sampled from SFB (37° 93.2'N, 122° 41.9'W) were primarily from Castro Rocks, located near the Richmond-San Rafael Bridge (Fig. 1). Castro Rocks is the largest haul-out site in northern SFB, most likely due to being one of the few sites accessible during low tide (Green and Grigg 2006). The most southern location, Channel Islands (CI; approximately 34° 03.9'N, 120° 37.4'W), comprises the three most northerly islands of the Channel Island National Park (Fig. 1). We collected blood from seals on Santa Cruz Island and San Miguel Island. These islands are situated far from human disturbance (~30 – 80 km from the mainland), and seals here tend to consume prey more representative of oceanic

species. Water depth in potential foraging locations is much deeper here than at the other locations, ranging from 20 to 230 m directly adjacent to shore, with depths reaching > 500 m, past the maximum diving threshold of harbor seals. Populations at both TB and CI are considered at carrying capacity and support healthy, sustainable populations of harbor seals.

Compound-specific isotope analysis of amino acids. The CSI-AA protocols used in this study have been recently described elsewhere (Germain et al. *in review*, Sherwood et al. 2011). Briefly, samples were first hydrolyzed with 6N hydrochloric acid followed by derivatization with triflouroacetyl/isopropyl esters, based on a modified protocol of Silfer et al. (1991). Individual amino acid $\delta^{13}C$ and $\delta^{15}N$ values were measured on a gas chromatograph coupled with an isotope-ratio-monitoring mass spectrometer (GC-IRMS; Thermo Trace Ultra GC linked to a Finnagin Delta^{Plus} XP mass spectrometer). Detailed derivatization chemistry and instrument settings are found in Germain et al. (*in review*). For $\delta^{13}C$-AA analysis (Table 1 and Supplementary Table 1), samples were injected in triplicate, and measured $\delta^{13}C$ values were corrected based on results from an external standard using the method of Silfer et al. (1991), which accounts for both carbon added and also fractionation during derivatization. For $\delta^{15}N$ analysis (Table 2 and Supplementary Table 2), samples were injected in quadruplicate. Measured $\delta^{15}N$ values were compared to results for repeated injections of an external AA lab standard. Individual $\delta^{15}N$-AA values were corrected if the average external

standard values for a given run deviated from known standard values by more than one standard deviation (Lehman et al. 2010). Isotopic results are expressed in parts per thousand (per mil, ‰) 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.

Figure 2 shows typical GC trace spectra for carbon and nitrogen for a harbor seal blood sample. The AAs measured were: alanine (Ala), aspartic acid (Asp = Asn + Asp), glutamic acid (Glu = Gln + Glu), isoleucine (Ile), leucine (Leu), proline (Pro), valine (Val), glycine (Gly), lysine (Lys), serine (Ser), threonine (Thr), tyrosine (Tyr), phenylalanine (Phe), and arginine (Arg). Ile typically had co-elution problems with Leu, especially due to its very small peak size.

Amino acid groupings: In figures and text, we organize both $\delta^{13}C$-AA and $\delta^{15}N$-AA data into several separate groupings. We used three groupings for $\delta^{13}C$ values (following O'Brien et al. 2002): essential (indispensable) AAs (E-AA: Val, Leu, Ile, Phe, Thr, Tyr, Arg), conditionally essential AAs (CE-AA: Pro, Ser, Lys), and non-essential (dispensable) AAs (NE-AA: Ala, Gly, Asp, Glu). For nitrogen, AA values were grouped as trophic AAs (Ala, Asp, Glu, Ile, Leu, Pro, Val), source AAs (Gly, Lys, Ser, Tyr, Phe, Arg), and metabolic AAs (MB: Thr). The trophic and source AAs are now standard groupings (McClelland and Montoya 2002,

Popp et al. 2007, Chikaraishi et al. 2009), while the MB designation for Thr was recently proposed by Germain et al. (*in review*) as discussed below.

Trophic position estimation and statistical analysis. Harbor seals were grouped based on sampling location and age class before calculating their average trophic position (TP) values. Both δ^{15}N-AA values and also bulk δ^{15}N values were used to calculate trophic position. Because recent work has indicated that different TEF values exist for urea excreting vs. ammonia excreting organisms (Germain et al., *in review*), two independent formulations for CSI-AA TP calculations were used. Both formulations use the AAs Glu and Phe, which are generally accepted as best trophic and source AAs, respectively. The "Single-TEF" equation is based on the standard approach that is currently used widely, and is derived from McClelland and Montoya (2002):

(1) $TP_{Single\text{-}TEF} = [(\Delta^{15}N_{(Glu\text{-}Phe)Seal} - 3.4) / 7.6]) + 1$

where 3.4 represents $\delta^{15}N_{Glu}$- $\delta^{15}N_{Phe}$ in primary producers; 7.6 represents $TEF_{Glu\text{-}Phe}$ for ammonia-excreting organisms; and +1 is to account for one trophic step. In contrast, the "Multi-TEF" approach, which has been hypothesized as more appropriate for marine mammals (Germain et al., *in review*), uses $TEF_{Glu\text{-}Phe}$ of 7.6‰ for all seal prey (ammonia-excreting), but a lower $TEF_{Glu\text{-}Phe}$ of 4.3‰ for the final tropic transfer to marine mammals (urea-excreting):

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

Derivation of this equation is detailed in Germain et al. (*in review*). Briefly, however, $\Delta^{15}N_{Glu\text{-}Phe,Seal} = \delta^{15}N_{Glu} - \delta^{15}N_{Phe}$ measured in the seal serum; both TEF values of 4.3‰ (urea) and 7.6‰ (ammonia) are represented; and +2 is to account for two trophic steps.

TP estimates were also made based on bulk $\delta^{15}N$ measurements ($\delta^{15}N_{Bulk}$) of seal serum, again in two ways. First, we used a standard calculation, as presented in Germain et al. (2011):

(3) $TP_{Bulk,Literature} = [(\delta^{15}N_{Bulk} - 3.8) / \delta^{15}N_{Plankton,Literature}]) + 2$

where $\delta^{15}N_{Bulk}$ is the measured bulk serum value; 3.8 represents $\Delta^{15}N$ for one trophic transfer from seal to prey (Germain et al. 2011); $\delta^{15}N_{Plankton,Literature}$ is the assumed $\delta^{15}N$ value at the base of the food web derived from literature sources; and +2 is to account for two trophic steps. An alternate calculation based on bulk $\delta^{15}N$ values instead uses the measured the $\delta^{15}N$ value for Phe, instead of literature values, as a proxy for the $\delta^{15}N$ value at the base of the food web:

(4) $TP_{Bulk,Phe} = [(\delta^{15}N_{Bulk} - 3.8) / \delta^{15}N_{Phe}]) + 2$

where $\delta^{15}N_{Phe}$ is the measured $\delta^{15}N$ value for Phe, averaged for all seals from a given location.

RESULTS AND DISCUSSION

Harbor seal food sources can be highly varied, but might be expected to vary both with oceanographic regime (i.e., population location), and also with age

class. Harbor seals spend a majority of their day on haul-out sites, which are typically mud flats, sandy and rocky beaches, either for resting, molting, pupping or thermoregulation (Allen 1991). They prefer haul-out sites with close proximity to water and prey resources and minimal disturbances. Since they tend to forage near their haul-out locations, their diet will reflect locally available prey. Harbor seals are highly opportunistic feeders, foraging primarily in shallow waters on benthic and small epibenthic fish ~10-30 cm in length and on invertebrates (Iverson et al. 1997). However, younger seals typically forage relatively closer to their haul-out sites, and travel shorter distances and to shallower depths vs. their older counterparts. Both carbon and nitrogen isotope values might be expected to vary related to both environment and age class.

Essential AA $\delta^{13}C$ patterns and values

Figure 3 presents $\delta^{13}C$-AA values for different seal age classes (> 2 yr = A/SA; < 2 yr = Y/W) for each of our three sampling locations. The essential AAs should allow a direct examination of relative carbon sources for different ages and populations of harbor seals, since they undergo minimal carbon isotope fractionation with trophic transfer (e.g., O'Brien et al. 2002, McMahon et al. 2010). One might expect that $\delta^{13}C$ values at the base of the food web in SFP, CI and TB might differ, and within a region, if younger vs. older seals feed on prey items that obtain carbon from different food webs. Such differences among and within populations should be revealed by carbon isotope analysis of E-AAs.

At the most oceanic CI site (n=9), the $\delta^{13}C$ E-AA values of younger seals are generally higher than those for older seals (Fig. 3a; excluding Thr due to unexpected enrichment and Ile due to chromatography issues, as discussed above; Fig. 2). This difference is also reflected in the average $\delta^{13}C$ values for all E-AAs (star symbols in Fig. 3); older seals have mean values (± one standard deviation) of -22.0 ± 2.3‰, vs. -21.0 ± 2.6‰ for younger seals. While the mean differences are not statistically significant, their consistency among all the individual E-AAs suggests that the result is real, and that younger seals are feeding in a food web that is ^{13}C-enriched relative to that supporting older seals. One possible explanation is a difference in food webs based on kelp/benthic vs. plankton primary production. Kelp is far more ^{13}C-enriched than marine plankton (Foley et al. 2010), therefore prey deriving more carbon from kelp would be expected to have substantially ^{13}C-enriched E-AAs. Alternatively, even in plankton, near-shore ecosystems tend to be slightly ^{13}C-enriched relative to off-shore/pelagic systems (Graham et al. 2007).

Another possibility relates to differences in diet quality. A recent experiment on tilapia reveals that when animals are maintained on low protein diets, carbon from carbohydrates or lipids may enter the indispensible pool via microbial synthesis in the gut (Newsome et al 2011). Whether or not such affects occur in mammals is not yet known. Further, for high protein diets (45% or greater), the microbial contribution was predicted to be small (less than 10-20%).

Pinniped diets are so rich in protein that such processes are unlikely to complicate interpretations of E-AA data. Consequently, the most likely explanation for differences in $\delta^{13}C$ E-AAs values between younger and older CI harbor seals is near-shore vs. offshore foraging, respectively, which fits the known foraging behaviors of these age classes.

The SFB seals (n=15), in contrast, exhibit minor d13C differences between age classes for most E-AAs (Fig. 3b), with the exception of Tyr and Arg, which are specifically required in growing animals (Rogers et al. 1970). The average of all $\delta^{13}C$ E-AA values in older seals is -22.0 ± 1.8‰, whereas younger seals have a slightly higher value (-21.2 ± 2.2‰). However, even this small offset in mean values is likely only due to differences in Tyr and Arg; based on the other AAs, the averages would be indistinguishable. This suggests that, unlike at CI, different age classes of seals in SFB are most likely feeding on prey with similar ultimate carbon sources. This seems consistent with the more restricted SFB habitat, such that one might expect the same sources of primary production for all prey available to SFB seals, which are made up of similar benthic and epibenthic fish and invertebrates (Torok 1994). While we cannot currently explain the differences in age classes for Tyr and Arg, the fact that all other $\delta^{13}C$ E-AA values are similar suggests an overall similarity of carbon sources for all seal prey. Given the increased demand for Tyr and Arg have been liked to rapidly growing animals, it

is possible the fractionation observed in these AAs might be related to a weaning effect in young animals.

Non-Essential AA $\delta^{13}C$ patterns and values

In contrast to E-AAs, the $\delta^{13}C$ values for CE-AAs and NE-AAs are fundamentally related to the degree of *de novo* synthesis for each AA (Howland et al. 2003, McMahon et al. 2010, Newsome et al. 2011). However, since different major biochemical classes (e.g., carbohydrates, proteins, lipids) may have different $\delta^{13}C$ values, even in the same prey item (e.g., lipids are ^{13}C-depleted) (Hayes 2001), the NE-AA values have also been linked to relative composition of diet. Specifically, in animals that consume lower protein diets, the $\delta^{13}C$ NE-AA values in body tissues can differ substantially from the same NE-AAs in diet due to incorporation of carbon from dietary carbohydrate or lipid, whereas in animals that consume high protein diets, $\delta^{13}C$ NE-AA values are more similar between diet and animal tissues, since it is proposed to be more energetically favorable to route AA directly from diet rather than biosynthesis under these conditions (Ambrose & Norr 1993, Tieszen & Fagre 1998, Jim et al. 2006).

At CI most CE-AAs and NE-AAs show little variation between age classes (Fig. 3). The average of all $\delta^{13}C$ CE-AA and NE-AA values in older seals is -12.1 ± 1.5‰, and younger seals have a very similar value of -12.5 ± 1.5‰ (excluding Gly from particularly high offsets). In particular, Ala and Gly had substantial

offsets between age classes. Together, this would seem to support the interpretation from the E-AA discussed above, that NE-AA routing is high (from a high protein diet) and that to the extent that non-protein is being taken up in diet, those $\delta^{13}C$ values are not all that different from mean NE-AAs.

At SFB, some CE-AAs and NE-AAs show substantial fractionation between age classes. For all CE-AA and NE-AA, the average $\delta^{13}C$ in older seals was -11.6 ± 2.0‰, while in younger seals it was slightly lower at -12.6 ± 2.1‰, whoever Ser, Asp, and Glu were substantially fractionated. Ser was offset by -6.0‰ in the older seals compared to the younger, while Asp was +4.1‰ and Glu was +4.9‰ higher in the older seals. Availability of Ser (a CE-AA required for normal growth) has been linked to the ability to recover from illness or injury (Young and El-Khoury 1995, Reeds 2000). Therefore, if the strong fractionation in this AA in younger seals is linked to Ser depletion in diet, it might suggest these animals are prone to nutritional stress. Both Asp and Glu are intermediates in the Krebs cycle, and we theorize a primary reason for ^{13}C-depletion in these AAs in younger seals may be that these animals had weaned recently. Since seal milk is rich in lipids, which have much lower $\delta^{13}C$ values than protein and carbohydrates (DeNiro and Epstein 1977), incorporation of carbon from milk lipids might lower $\delta^{13}C$ NE-AA values in nursing or recently weaned animals. While we tried to sample fully weaned animals in all locations, the SFB population weans later in the year than the CI population. Given our sampling schedule, SFB seal blood was

collected closer to the date of weaning than CI seal blood. Thus, we might expect that the recent lipid-rich diet would have a greater impact on the SFB population than the CI population. In any case, when coupled with the results from E-AAs (which suggest that both younger and older SFB animals obtain carbon from the same food web), our results for NE-AAs suggest they might be sensitive isotopic indicators of nutritional stress or nursing/weaning, producing a ^{13}C-depleted biochemical 'milk' signature.

Finally, the seals in TB (n=3) exhibited offsets in both the E-AAs and NE-AAs between age classes (Fig. 3c), which might suggest that young vs. old seals in this location seals are feeding on prey with different sources of primary production, and also with experience different levels of AA routing. However, with such a small sample set, it seems unwise to propose any detailed analysis of data from this location.

$\delta^{15}N$ patterns and values of individual amino acids

Figure 4 plots $\delta^{15}N$-AA values, normalized to Phe, for seal age classes (> 2yr and < 2yr) at CI and SFB (TB data are not shown because there were only two seals, both less than a year in age, however there were three seals for ^{13}C). As noted in the *Introduction*, $\delta^{15}N$-AA values can identify an animal's trophic position, while simultaneously providing an estimate of $\delta^{15}N$ values at the base of the food web (Martinez del Rio et al. 2009). We apply normalization to facilitate intercomparison of $\delta^{15}N$-AA patterns for seals from different environments. We

normalized data for each site to Phe since it is the most reliable source AA, remaining essentially unchanged through multiple trophic transfers, and therefore closely approximating $\delta^{15}N$ values at the base of the food web (Chikaraishi et al. 2009, Germain et al. *in review*, Styring et al. 2009). After this normalization, possible variation in primary production $\delta^{15}N$ values between CI and SFB environments should be removed, and remaining differences should be interpretable in terms of trophic structure differences.

In CI (Fig. 4a), the source $\delta^{15}N$-AA values were essentially the same for both age classes, as would be expected if all seals had similar N sources. The majority of the trophic AAs, however, were ^{15}N-enriched in the older seals. The difference between the average trophic and source AA is likely the broadest, most intercomparable measure of relative trophic position (Sherwood et al. 2011). If we take the difference between the main trophic (Glu, Ile, Leu, and Pro) and source (Gly, Ser, and Phe) AAs, the older seals in fact have a higher trophic-to-source difference (13.1 ± 1.1‰) vs. younger seals (11.3 ± 1.8‰). This suggests that older seals in CI are feeding at a somewhat higher trophic position than their younger counterparts. This is consistent with the interpretation drawn from $\delta^{13}C$-AA data, that older CI seals feeding on different prey, most likely more larger, more oceanic fish.

In constrast, seals in SFB again show the opposite relationship between age classes. The older seals in SFB have a trophic-source difference of 10.0 ± 1.6‰,

while the younger seals have a difference of 11.9 ± 1.7‰ (Fig. 4b). The higher $\delta^{15}N$ values for the younger seals indicate a generally *higher* trophic position. This result seems highly unlikely given expectations from prior studies on seal behavior and ecology. However, one explanation for an apparently "inverted" trophic position vs. age would be a "milk artifact", such that younger seals at the time of sampling were only recently weaned, and therefore still exhibited ^{15}N-enriched values (Kurle and Gudmundson 2007). As noted above, SFB seals were in fact sampled close to time of expected weaning (late May and early June), approximately a month after birth. If pups solely consumed their mother's milk, they would be expected to have elevated trophic $\delta^{15}N$-AA values compared to their mother's tissue. In SFB, the younger seals have average trophic $\delta^{15}N$-AA values elevated by 2‰ vs. older seals. Since the isotopic turnover time of serum is ~ 2 to 3 weeks, this intermediate value indicates partial isotopic equilibrium, suggesting weaning occurred within several weeks of sampling.

Nutritional stress on younger SFB seals should also be considered as an alternate explanation. Animals that are profoundly stressed (*i.e.,* starving or in negative N balance) catabolize their own body proteins to maintain essential functions, which can lead to dramatic ^{15}N-enrichment of remaining body tissues (Katzenberg and Lovell 1999, Fuller et al. 2005). Overall, however, there are no data suggesting widespread starvation of young seals in SFB (Green and Grigg

2006). Further, the milk artifact explanation would also be consistent with the $\delta^{13}C$ NE-AA data for SFB seals discussed above.

Finally, Thr is a unque AA in terms of its nitrogen isotope systematics. This AA has been designated as a "metabolic AA," because it is the only AA to become strongly ^{15}N-depleted in many higher TP animals (Hare et al. 1991, Styring et al. 2009). As expected, Thr is very strongly ^{15}N-depleted in all seals at both locations (Fig. 4a,b). The relative ^{15}N-depletion of Thr in these wild seals (vs. Phe) is among the largest reported in any animal, consisent with the hypothesis that Thr may be exceptionally ^{15}N-depleted in marine mammals (Styring et al. 2009), perhaps linked to bioichemical pathways involved in blubber formation (Germain et al. *in review*). However, in our data there is also a consistent difference associated with age class; Thr $\delta^{15}N$ values are ~2-3‰ lower for younger vs. adult seals in both locations. To our knowledge this is the first time a clear link to age or growth status has been documented, and it may provide insight into the poorly understood mechanisms for Thr ^{15}N-depletion. Based on early CSI-AA work, it has been suggested that Thr ^{15}N-depletion could be an indicator of nutritional stress (Hare et al. 1999). In truly starving animals, this could be reasonable, if catabolic breakdown of Thr to supply energy (e.g., Champe et al. 2008) preferentially liberated ^{15}N-enriched Thr from skeletal muscle into the exogenous waste products, leaving lighter ^{14}N behind. The biochemical mechanism favoring the liberation of ^{15}N-enriched Thr has never been identified,

however, and run counter to most biochemical processes, where bonds involving light isotope tend to be less stable than those involving heavy isotopes. Further, as noted above, there is no indication that larger proportions of the younger animals at either SFB or CI are starving. Finally, the fact that Thr depletion occurs in younger animals at *both* sites, including the CI site with a healthy and growing population, seems to rule out this as an explanation. Instead it suggests that the additional ^{15}N-depletion in Thr in younger animals is related to rapid growth. This set of observations reinforces the idea that Thr is a unique AA, for which changes in ^{15}N values are unrelated to either trophic or source groupings, but instead are an indicator of relative metabolic activity in an organism.

Estimates of trophic position via δ^{15}N-AA values

One of the main applications proposed for CSI-AA data is the potential to calculate trophic positions (TP) with far more accuracy than has previoulsy been possible based on either bulk isotopes or gut content analyses (Gibble 2011). However, we have recently proposed that a new "multi-TEF" approach is required for marine mammals, which in contrast to the traditional "single-TEF" CSI-AA calculation, takes into account hypothesized major differences in nitrogen fractionation in ammonia vs. urea excreting animals (Germain et al., *in review*). Using our CSI-AA data, we are able to test these ideas for the first time in a wild marine mammal population. We calculated trophic positions for each location (CI, SFB, TB) and age class (A, SA, Y, W) using both a traditional single–TEF

approach and the new proposed multi-TEF formulaltion (Fig. 5a). Furthermore, we compare these values to TP estimates based on bulk isotope values, and also literature estimates based on scat analysis (Fig. 5b).

Using both the single-TEF and the multi-TEF CSI-AA approaches, identical trends with age class are observed at all sites (Fig. 5a). However, the single-TEF approach (equation 1; McClelland and Montoya 2002) returns TP estimates that are consistently ~0.5 lower than the mult-TEF approach (equation 2; Germain et al. *in review*). While this absolute TP offset is not large, comparisons with independent estimates suggest that the multi-TEF approach is more accurate. Specifically, the range of values for the mult-TEF CSI-AA approach falls directly in line with the ranges expected from scat analysis (Fig.5a).

The CSI-AA approaches offer a view of trophic structure for different seal age classes that is consistent among locations, with the interpretation of $\delta^{13}C$ NE-AA values offer, and with basic ecological inferences about how trophic level changes with age. In both the CI and TB, CSI-AA estimates indicate a clear trend of TP increasing with age. In particular for CI, CSI-AA estimates suggest a remarkably consistent relationship of increasing TP with age (~0.4 TP from weaners to adults), while TB suggests a greater difference (~0.7 TP) from weaners to adult seals). Conversely, the SFB seals have a different pattern than the outer coastal sites, where the younger seals (weaners and yearlings) are feeding at *higher* trophic positions than the adults and subadults (Fig. 5a). For weaner seals, this

result is consistent with the milk artifact hypothesis above. However, the SFB yearlings also displayed unexpectantly high TP values. This could be interpreted as suggesting nutritional stress in this age class, especially since $\delta^{13}C$ E-AA values showed that all SFB seals are feeding in the same food web. We note that TP of seals consuming different prey species would range from about two to four trophic positions, exactly the range indicated by the multi-TEF equation (Fig. 5a).

Finally, the overall utility of CSI-AA values for TP estimations in these wild populations can be evaluated by comparing CSI-AA TP estimates with those derived from the more traditional, bulk isotope $\delta^{15}N$ approach. These results (Fig. 5b) illustrate the inherent limitations of bulk isotope approaches, and the potential of CSI-AA. TP estimates based on bulk isotope values (e.g., equation 3; Germain et al. 2011) require two fundamental assumptions: 1) the $\delta^{15}N$ value of primary production at the base of a food web, and 2) the change in bulk $\delta^{15}N$ values with each trophic transfer between consumer and food ($\Delta^{15}N$). While the latter is somewhat variable, there is a large amount of data available to provide confidence in $\Delta^{15}N$ values. However, the *average* $\delta^{15}N$ at the base of the food web for a given location or species may be impossible to measure directly, and therefore an estimate must be made based on literature values involving significant assumptions. Using limited available literature values for $\delta^{15}N$ value of phytoplankton in all regions ($\delta^{15}N_{Plankton}$ in TB = 7.5‰, Rau *et al.* 1998; SFB = 8‰, Cloern *et al.* 2002; CI = 6.5‰, Germain et al., unpublished data), bulk $\delta^{15}N$

TP estimates are significantly higher than *both* CSI-AA and scat analysis estimates of TP. This indicates that available literature values differ substantially from the actual *average* $\delta^{15}N$ values of primary producers. Finally, there is also greater variability in TP and no discernable trend with age class, suggesting that with bulk $\delta^{15}N$ values, this information has been lost.

Since the $\delta^{15}N$ value of Phe is hypothesized to be closest to the approximate $\delta^{15}N$ value at the base of food web, using the measured Phe $\delta^{15}N$ value (instead of assumed literature values) allows a direct test of the hypothesis that bulk TP estimates are in error primarily from the inability to accurately estimate source values. In this study, Phe values were elevated and varied slightly at each location, as would be expected based on the different oceanic environments (14.4‰ at CI, 13.4‰ at SFB, and 12.2‰ at TB). These elevated values are likely influenced by upwelled water from the California counter current (CCC), which carries nitrate that is substantially ^{15}N-enriched due to denititrification northward along coastal CA (denitrification can enrich the ^{15}N in the upwelled nitrate to ~15‰ to 20‰) (Altabet 1999). The $\delta^{15}N$ values most likely reflect this process, however, it is also possible that additional terrestrial or anthropogenic inorganic N sources, or an altered N cycle associated with more eutrophic environments (especially in SFB) might further increase $\delta^{15}N$ values. In any case, using these Phe $\delta^{15}N$ values as a direct proxy for average primary production in the bulk $\delta^{15}N$ TP calculation (equation 4) results in substantailly reduced TP estimates, all now

within the range expected from both independent scat analysis and CSI-AA (Fig. 5b). Together, these comparisons illustrate how CSI-AA data can overcome some of the major inherent limitations in bulk isotope anslysis, and also suggest that CSI-AA can reveal "fine structure" in relative TP values in an ecosystem, that may be obscured using either bulk isotope or traditional stomach or scat analyses.

CONCLUSIONS

This study demonstrates that compound-specific isotope analysis of amino acids is a powerful tool for applied ecology, with the potential to greatly refine predictions of an animal's foraging ecology and dietary behavior. Groupings of AAs were consistent with previous studies: essential, conditionally essential, and non-essential AAs for $\delta^{13}C$ values; and trophic, source and metabolic for $\delta^{15}N$ values. Thr was once again greatly ^{15}N-depleted compared to all other AAs. It appears to be consistently ^{15}N-depleted in marine mammals, but also could be a potential indicator of extreme growth in mammals. The $\delta^{13}C$-AA and $\delta^{15}N$-AA values and patterns were vital in examining differences between older and younger harbor seals in several CA locations. Specifically, SFB shows surprising results, where both the $\delta^{15}N$-AA values and unexpectantly high TP estimates in the yearling populations, suggest nutritional stress. In contrast, the weaners in SFB may have only recently weaned from their mother's milk, showing high $\delta^{15}N$ values due to a milk artifact. The importance of CSI-AA is especially apparent

when using a multi-trophic enrichment factor equation (Germain et al., *in review*), which yielded reasonable TP estimates in our harbor seals compared to previous bulk trophic position calculation methods, where values were independent of age class and variability was greater. Future detailed, direct study on animals under definite nutritional stress from poor diet with a large group of animals is needed to explore the expression of nutritional stress in CSI-AA values (e.g., elephant seals and other mammals that naturally lose mass during lactation or hibernation periods). Predicting the health status of mammals using this CSI-AA non-invasive method has significant potential in conservation management of threatened or endangered species.

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					δ^{13}C (‰)														
Sample ID #	Fraction	Sex	Age	Loc	Ala	Gly	Thr	Ser	Val	Leu	Ile	Pro	Asp	Glu	Phe	Lys	Tyr	Arg	Bulk
1546	RBC	M	A	CI	-19.4	0.1	-6.9	3.2	-20.4	-17.9	10.0	-15.8	-11.3	-11.4	-22.0	-16.4	-23.8	-22.3	-15.9
1502	RBC	M	Y	CI	-10.2	-6.3	-6.3	-1.0	-23.1	-19.2	-29.7	-14.6	-16.6	-16.0	-23.3	-17.5	-24.1	-21.6	-16.5
1458/59	RBC	F	A	SFB	-12.8	-2.3	-5.3	-1.1	-18.7	-23.6	-14.5	-16.5	-15.1	-13.8	-23.2	-15.9	-22.1		-16.3
1580/81	RBC	M	Y	SFB	-18.7	-0.2	-7.4	6.4	-22.2	-20.5	18.8	-16.2	-17.0	-14.4	-23.9	-17.1	-23.3	-22.5	-16.0
1547/51	RBC	M	W	SFB	-16.3	-4.3	0.7	-0.1	-17.7	-16.7	11.7	-15.5	-10.9	-11.3	-22.9	-17.9	-24.4	-20.0	-16.8
1546	Serum	M	A	CI	-17.0	1.8	-2.8	1.4	-14.4	-23.0	-9.6	-11.2	-7.1	-11.0	-27.0	-17.1	-24.6	-15.0	-16.2
1503	Serum	M	SA	CI	-16.5	-17.5	-1.4	1.0	-22.1	-25.8	-19.0	-16.8	-14.0	-11.9	-26.7	-19.4	-32.8	-15.6	-16.6
1517	Serum	F	SA	CI	-11.0	-0.2	-3.2	-8.5	-17.9	-23.3	-13.5	-12.7	-12.1	-16.3	-26.1	-17.1	-21.9	-13.3	-16.7
1498	Serum	F	Y	CI	-14.6	0.6	-7.3	1.2	-16.6	-24.9	-17.1	-17.0	-5.8	-15.4	-26.1	-18.6	-22.7		-17.0
1502	Serum	M	Y	CI	-16.2	-10.7	-3.9	-10.1	-16.1	-23.7	-12.7	-15.2	-9.7	-12.4	-25.7	-16.0	-21.0	-21.3	-16.9
1489	Serum	F	W	CI	-14.7	0.4	-2.5	6.3	-14.5	-23.2	-14.3	-14.1	-7.9	-13.6	-24.6	-17.0	-23.5	-4.3	-15.8
1492	Serum	M	W	CI	-21.0	25.5	-4.3	-16.7	-21.8	-19.1	-28.8	-4.8	-12.4	-13.6	-29.1	-16.8	-30.1		-17.6
1490	Serum	F	W	CI	-20.1	-3.3	-8.9	3.8	-13.9	-23.5	-15.6	-13.1	-13.2	-13.6	-24.1	-16.4	-20.2	-13.6	-16.5
1542/43	Serum	F	W	CI	-20.4	-5.8	-3.5	-9.5	-13.0	-21.3	-9.1	-13.8	-10.3	-12.3	-25.6	-15.8	-19.8		-17.1
1578/79	Serum	F	A	SFB	-12.7	4.6	-1.5	-1.1	-15.4	-24.1	-15.2	-9.3	-6.1	-6.2	-23.3	-14.9	-23.6		-16.0
1458/59	Serum	F	A	SFB	-17.2	-7.4	-7.1	-9.7	-16.0	-22.4	-11.8	-13.7	-9.6	-12.2	-22.3	-16.8	-27.6	-16.4	-16.0
1460/61	Serum	F	A	SFB	-14.4	2.0	-2.3	1.0	-14.3	-20.8	-24.6	-17.3	-15.5	-6.7	-20.6	-12.3			-15.7
1445/1588	Serum	M	SA	SFB	-13.5	6.5	-8.3	-9.2	-15.7	-22.4	-16.6	-12.0	-6.7	-6.4	-29.6	-19.4	-26.7	-20.9	-17.2
1456/57	Serum	F	SA	SFB	-17.0	6.1	-1.1	-0.3	-19.6	-23.8	-15.6	-14.2	-5.6	-11.4	-26.1	-16.4			-17.0
1598/99	Serum	F	SA	SFB	-14.2	4.7	-5.1	-3.8	-21.0	-24.0	-21.6	-11.2	-16.2	-11.7	-26.4	-15.6	-24.6		-16.8
1580/81	Serum	M	Y	SFB	-17.1	6.1	-2.1	-4.4	-16.3	-24.2	-12.0	-14.8	-11.0	-12.2	-25.1	-16.6	-24.6	-14.5	-16.1
1590/91	Serum	M	Y	SFB	-20.3	-7.5	-12.5	-7.7	-25.5	-28.5	-34.7	-19.2	-18.3	-16.7	-26.8	-16.6	-25.0	-18.9	-17.3
1592/93	Serum	F	Y	SFB	-17.5	-4.4	-3.9	0.0	-18.5	-23.5	-17.9	-14.1	-13.8	-12.8	-22.2	-16.2	-22.6	-18.2	-15.8
1519/20	Serum	M	W	SFB	-18.6	13.1	-3.8	1.6	-15.7	-27.0	-19.3	-13.5	-10.4	-16.5	-27.2	-17.2	-15.8		-17.2
1574/75	Serum	F	W	SFB	-13.4		-4.4	4.7	-7.3	-21.2	-9.1	-13.3	-19.5	-13.3	-22.0	-15.9	-21.8	-15.6	-17.8
1594/95	Serum	M	W	SFB	-22.9	0.2	-4.0	3.7	-19.1	-24.6	-18.9	-18.2	-16.2	-14.9	-23.6	-18.9	-24.5	-19.0	-17.4
1596/97	Serum	F	W	SFB	-16.4	2.6	0.8	13.0	-12.7	-19.4	-10.7	-8.3	-14.3	-12.8	-23.3	-12.7	-21.9	-13.2	-17.0
1547/51	Serum	M	W	SFB	-12.8	-4.5	-3.3	2.4	-16.5	-25.8	-10.4	-13.6	-15.5	-17.4	-26.0	-19.1	-26.3	-18.4	-17.8
1576/77	Serum	F	W	SFB	-17.4	0.0	-12.4	-2.4	-11.8	-23.0	-16.0	-6.0	-11.0	-13.0	-22.7	-14.3	-22.5		-17.0
1450/51	Serum	F	Y	TB	-8.7	15.1	-6.4	-11.3	-21.6	-25.6	-31.8	-19.4	-2.4	-3.2	-34.1	-18.1	-24.5	-19.1	-16.8
1657/58	Serum	F	W	TB	-12.8	5.0	3.7	4.0	-15.9	-21.8	-14.3	-10.5	-11.0	-11.7	-25.1	-14.4	-22.8	-12.0	-15.6
CARBON ONLY																			
1542/43	RBC	F	W	CI	-18.4	5.2	-5.2	13.1	-24.9	-23.9	18.9	-19.5	-15.6	-9.8	-22.1	-15.7	-23.1		-15.9
1584/85	RBC	M	A	SFB	-18.1	-3.4	-7.7	-1.7	-20.0	-25.8	-26.4	-15.9	-17.3	-13.7	-23.3	-11.4	-23.2		-18.4
1659/60	RBC	F	A	TB	-15.8	-2.1	-8.1	-7.0	-18.8	-26.5	1.6	-14.7	-18.4	-15.1	-23.4	-13.7	-22.7		-15.9
1584/85	Serum	M	A	SFB	-17.3	-5.4	-7.6	-9.2	-19.7	-24.5	-12.7	-14.6	-16.1	-16.2	-24.5	-15.4	-22.8		
1586/87	Serum	M	W	SFB	-13.9	5.4	-1.5	2.6	-17.7	-26.8	-14.0	-11.9	-19.2	-20.9	-24.4	-18.4	-22.9		-17.7
1659/60	Serum	F	A	TB	-14.7	-4.4	-6.5	-7.3	-18.6	-26.6	-10.6	-13.5	-15.8	-15.3	-24.1	-15.2	-22.6		-16.2

Table 1. δ^{13}C-AA values from harbor seals off the California coast. δ^{13}C-AA values of wild harbor seals sampled in Spring of 2007, measured in both serum and red blood cell (RBC) fractions. Average analytical error (standard deviation) was ±0.5‰ across all AAs and samples. Abbreviations: Sex: Male (M), Female (F); Age: Adult (A), Subadult (SA), Yearling (Y), Weaner (W); Location (Loc): Channel Islands (CI), San Francisco Bay (SFB), Tomales Bay (TB). Blank fields indicate value was not determined (*see methods*).

					δ^{15}N (‰)															
Sample ID #	Fraction	Sex	Age	Loc	Ala	Gly	Thr	Ser	Val	Leu	Ile	Pro	Asp	Glu	Phe	Lys	Tyr	Arg	Bulk	TP
1546	RBC	M	A	CI	25.1	16.9	-21.2	18.0	26.3	27.1	18.7	24.8	20.9	24.3	14.2	12.2	9.4	16.7	17.4	2.3
1502	RBC	M	Y	CI	24.1	13.9	-16.8	14.6	25.0	25.1	24.4	21.6	19.7	24.0	12.3	6.4	11.9	10.7	15.8	2.5
1458/59	RBC	F	A	SFB	22.7	12.4	-14.9	12.2	21.5	24.3	25.4	22.9	17.0	23.2	11.8	2.2	10.7		16.0	2.5
1580/81	RBC	M	Y	SFB	22.6	12.5	-16.4	12.6	25.9	25.5	27.8	22.9	18.8	25.0	15.0	6.3	13.2	6.6	15.8	2.3
1547/51	RBC	M	W	SFB	25.3	15.5	-23.4	18.6	27.4	28.8	25.1	27.7	24.8	28.1	18.1	9.5	15.3	14.6	19.7	2.3
1546	Serum	M	A	CI	27.7	14.3	-26.8	17.5	29.5	27.5	30.4	34.0	22.1	29.5	16.1	11.1	16.3	20.8	19.2	2.7
1503	Serum	M	SA	CI	14.9	12.4	-13.9	11.7	20.8	24.1	23.6	25.9	19.8	25.7	12.3	15.0	14.9	24.3	17.8	2.7
1517	Serum	F	SA	CI	21.9	13.8	-16.4	13.4	21.8	25.7	25.8	27.8	19.4	26.0	15.0	6.1	13.2	8.4	16.6	2.4
1498	Serum	F	Y	CI	22.1	12.8	-19.9	11.8	21.3	24.6	25.0	25.3	19.8	24.7	13.7	6.7	13.0	12.9	16.6	2.4
1502	Serum	M	Y	CI	21.2	10.6	-18.0	11.8	24.1	22.3	22.4	21.9	18.6	20.3	10.3	9.3	16.5	15.6	16.1	2.3
1489	Serum	F	W	CI	25.9	16.9	-25.0	12.2	21.0	26.3	23.1	29.2	23.8	27.5	16.2	10.7	15.8	13.5	19.9	2.5
1492	Serum	M	W	CI	21.4	15.5	-14.9	17.7	20.5	23.4	24.3	26.0	20.5	25.4	16.7	17.6	11.9		19.3	2.1
1490	Serum	F	W	CI	25.0	16.7	-26.4	14.5	23.2	27.7	30.0	29.1	23.7	27.5	15.5	11.1	16.8	11.1	20.1	2.6
1542/43	Serum	F	W	CI	25.1	11.1	-27.7	15.5	26.0	26.4	26.8	28.7	23.2	21.7	13.4	9.1	13.2		20.9	2.1
1578/79	Serum	F	A	SFB	22.0	11.5	-15.5	12.5	18.0	23.0	17.9	23.6	18.1	25.5	12.8	6.5	10.8		15.8	2.7
1458/59	Serum	F	A	SFB	21.9	10.7	-16.5	12.7	25.9	24.8	25.5	25.0	19.8	24.4	14.0	11.4	16.0	16.8	16.2	2.4
1460/61	Serum	F	A	SFB	20.9	12.4	-11.2	8.4	17.8	20.6	18.2	24.7	16.2	15.6	12.2				15.9	1.4
1445/1588	Serum	M	SA	SFB	20.5	16.0	-14.6	14.7	22.7	22.7	20.2	26.7	18.9	23.2	14.5	8.5	14.2	16.1	17.2	2.1
1456/57	Serum	F	SA	SFB	22.3	14.4	-22.9	11.8	19.6	22.5	22.4	24.3	20.5	24.7	10.8	8.8		-3.0	16.2	2.8
1598/99	Serum	F	SA	SFB	22.4	15.8	-16.5	12.2	13.1	23.0	19.0	27.9	18.2	21.4	13.3	18.3	7.3		17.2	2.1
1580/81	Serum	M	Y	SFB	23.8	11.7	-20.0	14.6	25.4	23.5	23.8	26.7	19.6	26.1	14.4	10.9	13.5	15.4	16.9	2.5
1590/91	Serum	M	Y	SFB	22.9	12.9	-18.4	10.6	16.2	23.0	21.3	26.5	17.1	26.3	12.3	10.8	14.0	11.5	15.9	2.8
1592/93	Serum	F	Y	SFB	21.5	13.8	-13.2	12.4	17.0	24.2	25.0	25.5	16.4	23.9	15.3	4.2	12.1	10.7	16.3	2.1
1519/20	Serum	M	W	SFB	20.4	9.3	-20.9	15.5	19.8	22.5	21.6	28.2	17.7	22.6	11.9	7.9	14.9		17.7	2.4
1574/75	Serum	F	W	SFB	9.8		-13.2	5.4	8.9	19.2	12.4	26.4	19.7	23.1	16.1	15.4	14.7	22.1	18.5	1.9
1594/95	Serum	M	W	SFB	22.5	16.5	-16.2	12.8	17.9	23.6	23.1	29.8	18.0	23.2	12.8	14.9	14.0	14.7	18.3	2.4
1596/97	Serum	F	W	SFB	23.4	17.4	-16.2	8.9	17.5	27.0	24.1	31.1	23.0	25.8	8.5	8.2	19.9	10.8	19.1	3.3
1547/51	Serum	M	W	SFB	26.8	12.4	-23.0	19.5	28.3	26.7	27.3	32.9	22.4	27.6	17.9	13.3	17.0	22.9	20.1	2.3
1576/77	Serum	F	W	SFB	23.8	14.2	-19.0	13.7	19.3	25.6	30.4	28.9	20.9	24.7	14.0	5.9	11.3		18.7	2.4
1450/51	Serum	F	Y	TB	20.1	15.3	-19.0	13.9	23.9	22.1	19.5	28.2	20.2	24.3	9.9	6.4	15.5	14.5	16.4	2.9
1657/58	Serum	F	W	TB	21.0	14.8	-11.3	9.7	17.0	23.4	25.6	27.7	17.8	24.1	14.5	8.4	12.4	14.3	16.3	2.2

Table 2. δ^{15}N-AA values from harbor seals off the California coast. δ^{15}N-AA values of wild harbor seals sampled in Spring of 2007, measured in both serum and red blood cell (RBC) fractions. Average analytical error (standard deviation) was ±1.3‰ across all AA and samples. Sex, age, and location abbreviations as in Table 1. Trophic position (TP) was calculated using equation (1), as described methods. Blank field indicates value was not determined.

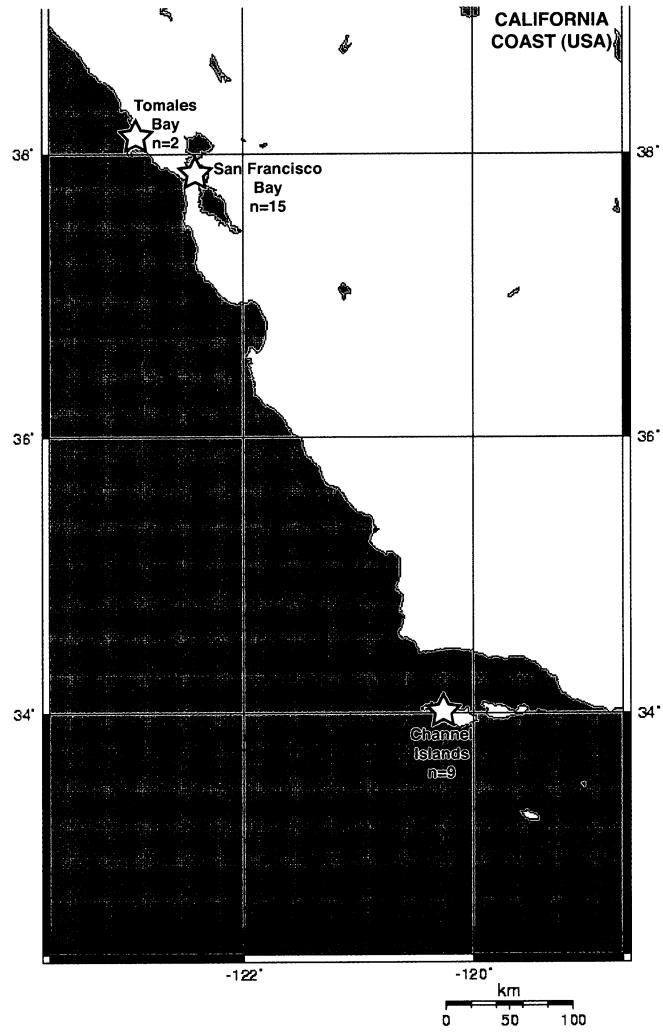


Figure 1. Sampling locations for wild harbor seals (*Phoca vitulina*). Blood samples were collected in the spring of 2007 from Tomales Bay, San Francisco Bay, and Northern Channel Islands. n = number of individuals from which blood was sampled at each location.

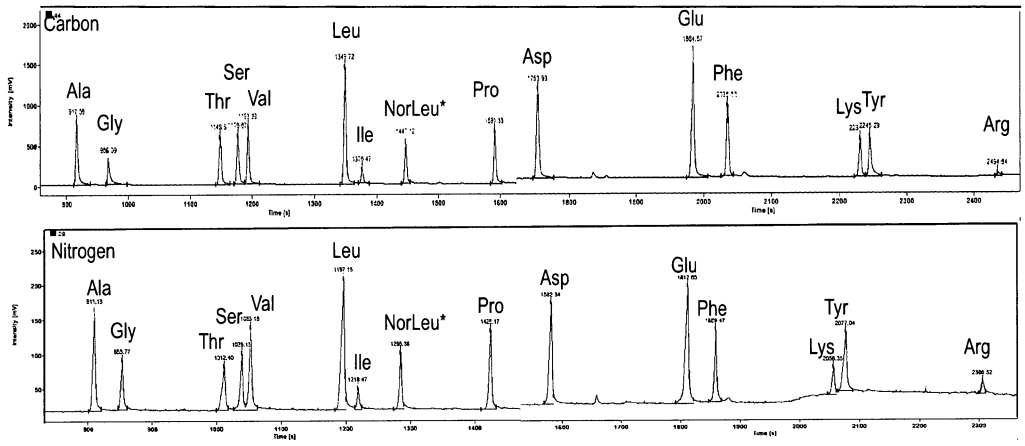


Figure 2. GC traces for all individual AAs for both $\delta^{13}C$ and $\delta^{15}N$ analyses. The panels show GC traces for the serum fraction, with retention time (min) on the x-axis and intensity (mV) on the y-axis. Chromatograms show the generally clean and excellent chromatography observed from blood serum hydrolysates. Traces are similar carbon and nitrogen, although peak fronting in the nitrogen trace is typical, due to relatively overloaded chromatographic conditions required, and shows decreased baseline separation between Ser/Val and Lys/Tyr. Individual AA abbreviations are defined in text; * refers to internal standard (NorLeu). Detailed methodology and instrument settings are described in Methods.

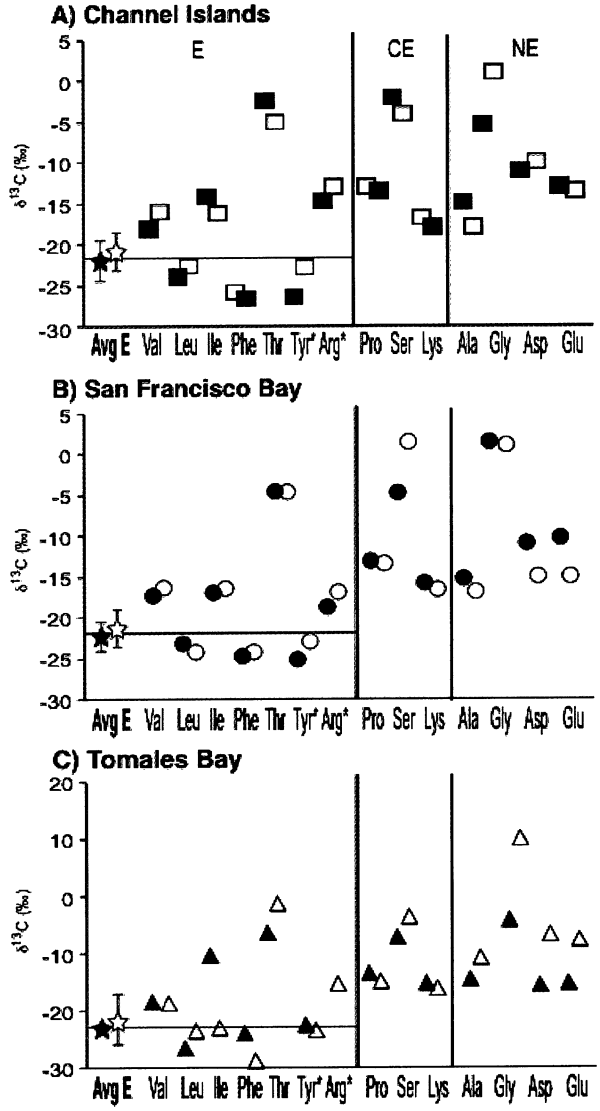


Figure 3. δ^{13}C-AA patterns in harbor seals from three locations in California. Measured (*i.e.*, non-normalized) individual AA data are arranged according to the essential (E), conditionally essential (CE) and non-essential (NE) groupings. A) Channel Islands (squares); B) San Francisco Bay (circles); C) Tomales Bay (triangles). For each location, results are plotted separately by age class (filled symbols > 2 yr = Adults/Subadults; open symbols < 2 yr = Yearlings/Weaners). Within the E-AA category, grey shaded area indicates one standard deviation around the mean (solid black line) for the average of all E-AAs measured (Val, Leu, Phe, Tyr, Arg) for all seals. Stars at left indicate the average values for each age class (filled star = > 2 yr; open star = < 2 yr; error bars indicate one standard deviation). Average analytical error for individual data points (±0.5‰; *Methods*) is smaller than symbol size. All AA abbreviations are defined in text; asterisks for Tyr* and Arg* indicate these E-AAs are needed in greater quantities in growing animals.

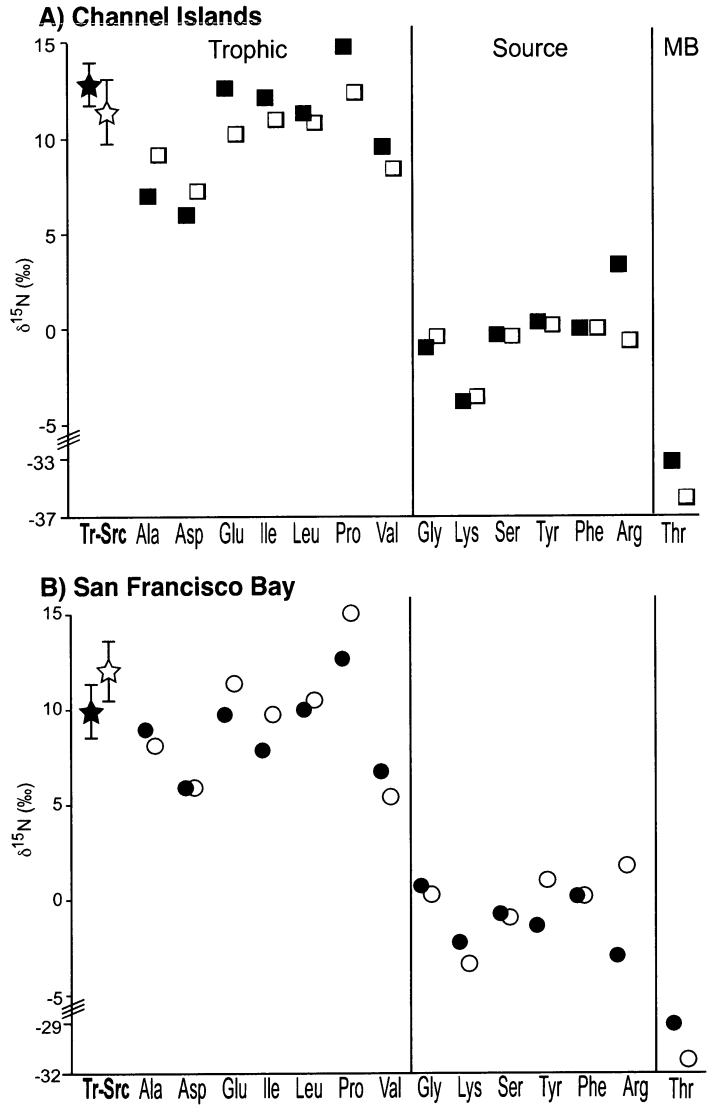


Figure 4. δ^{15}N-AA patterns in harbor seals from three locations in California. Individual AA data are normalized to Phe δ^{15}N to allow comparison relative patterns. AAs are arranged according to trophic, source and metabolic (MB) groupings. A) Channel Islands (squares); B) San Francisco Bay (circles). For each location, results are plotted separately by age class (filled symbols > 2 yr = Adults and Subadults; open symbols < 2 yr = Yearlings and Weaners). Stars at left indicate the *average* of "Trophic" AA values – *average* of "Source" AA values (Tr-Src) for each age class (filled star = > 2 yr, open star = < 2 yr), and error bars indicate one standard deviation. Average analytical error for individual data points (±1.3‰; *methods*) is similar to symbol size, and is not shown. All AA abbreviations are defined in text. Note scale break is required for inclusion of the extremely depleted Thr δ^{15}N values, and that Tomales Bay data is not included for δ^{15}N, because this location had only two animals < 2 yr.

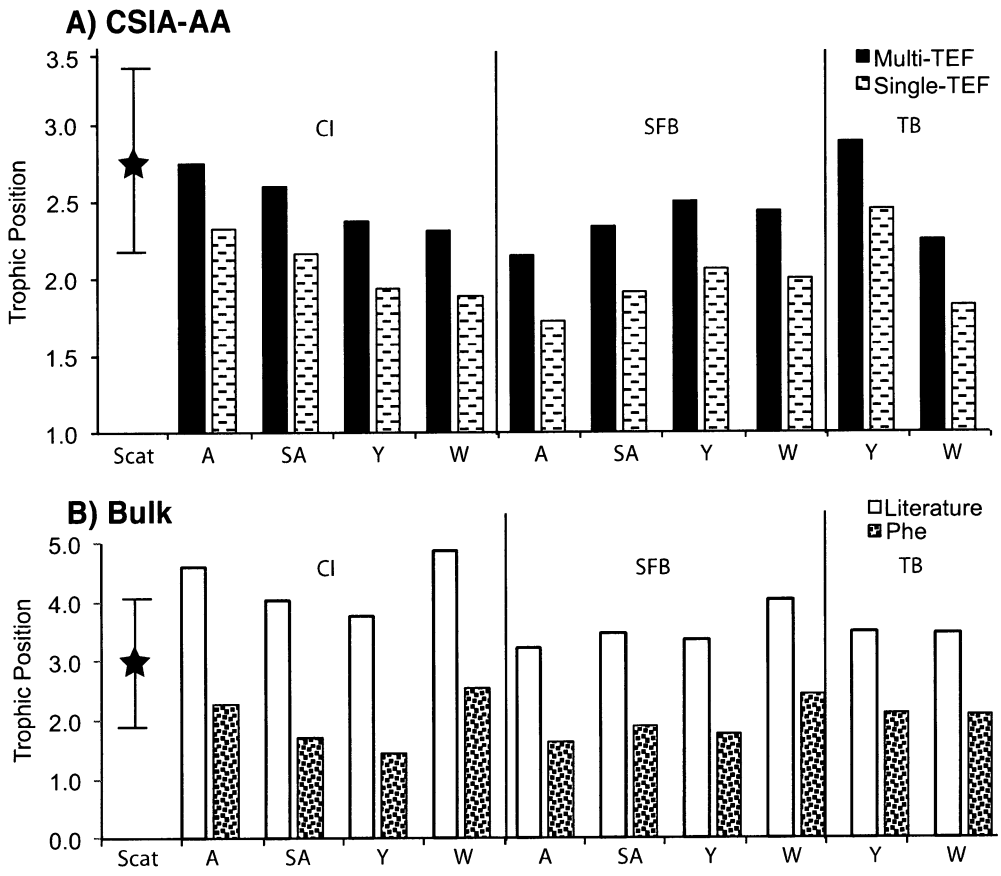


Figure 5. Comparative trophic position estimates using CSI-AA vs. Bulk $\delta^{15}N$. Average seal trophic positions from different calculation approaches, for each location (CI = Channel Islands; SFB = San Francisco Bay; TB = Tomales Bay) and age class (A = Adults; SA = Subadults; Y = Yearlings; W = Weaners). Star symbol at left indicates scat trophic position estimates from literature from similar CA regions (Tollit et al. 1997; Gibble MS thesis 2011; error bar = one standard deviation of literature estimates). A) CSI-AA based trophic position estimates compare results from the multi-TEF approach proposed for marine mammals (dark bars; Germain et al, *in review*) to traditional single-TEF results (light hatched bars). Specific equations used are given in *methods.* B) Bulk $\delta^{15}N$ trophic position estimates; all use a standard approach, and assume a 3.4‰ increase per trophic transfer. *Open bars* ("Literature") show results using an average $\delta^{15}N$ value for base of food web derived from literature. *Spotted bars* ("Phe") use the $\delta^{15}N$ value of Phe as direct proxy for $\delta^{15}N$ value at base of food web.

APPENDIX 1

Additional tables and figures for Chapter 1

Supplementary Table 1.1: $\delta^{13}C$ and $\delta^{15}N$ of all tissues (serum, muscle, blubber) for captive, rehabilitating seals at TMMC (LE = Lipid Extracted). Tissue was collected once, on final day in center either due to successful release, death, or euthanization (R; D; E). Discrimination factors ($\Delta_{tissue\text{-}diet}$) were determined from seals at TMMC for greater than 30 days (bolded). Health (1=emaciated; 7=obese). Refer to Supplementary Table 2 for specific tissue isotopic equilibrium graphs after grouping values from seals released or deceased over varying timeframes in TMMC.

Name	Sex	Age	Release/ Die/Euth	Day Tissue Collected	Health	Official cause of death
Melissa	F	P	R	**71**		
Nigel	M	P	R	**62**		
Hang Ten	F	P	R	**83**		
Luka	F	P	R	**93**		
Easter Egg	F	P	R	**83**		
Dicaprio	M	P	R	**79**		
Vendredi	M	P	R	**57**		
Degas	F	P	R	**93**		
Leopard	M	P	R	**71**		
Kirby	M	P	R	**61**		
Ladwig	M	P	D	**41**	4	Enteritis
BMB	F	P	D	**36**	3	Colitis
Skinny Dip	M	A	D	0	4	Shark Bite
Stefan	F	A	E	0	2	Pyothorax; Septicemia
Sharkie	M	P	D	**34**	3	Trauma (Bladder rupture)
Nabby	F	P	E	**62**	6	Neurological
Carcass	F	A	D	0	4	Bacterial Infection
Icey	F	P	D	16	3	Enteritis; Peritonitis
Cael	M	P	D	0	3	Pneumonia
Pinata	M	P	D	9	3	Enteritis; Peritonitis
Carcass	F	F	D	0	4	Trauma - Skull fracture
Rally	M	P	D	15	3	Phocine herpesvirus; Septice
Carcass	F	P	D	0	1	Fat atrophy
Sorenson	M	P	D	2	2	Fat atrophy
Stumpy May	F	P	E	18	3	Aspiration; Enteritis
Lord Stanley	F	P	D	3	1	Fat atrophy
Ding	F	P	D	7	3	Enteritis
Shenanigans	M	P	E	1	3	Trauma - Spine fracture
Carcass	M	W	D	0	4	Brain abscess
Sacs	F	P	E	1	2	Worms in Liver, Heart, Lungs
Carcass	-	-	D	1	-	-

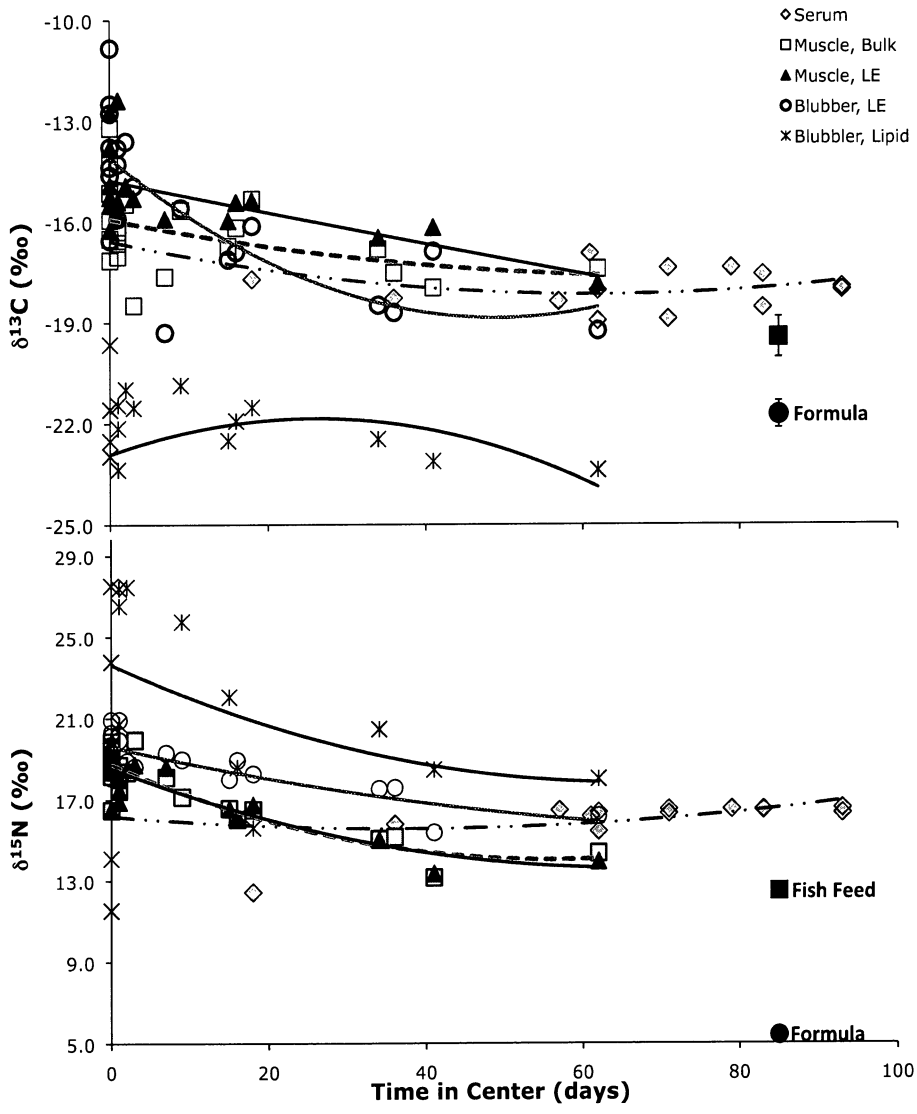
Supplementary Table 1.1 (cont):

Name	Serum		Muscle, Bulk		Muscle, LE		Blubber, LE		Blubber, Lipid	
	$\delta^{13}C$	$\delta^{15}N$	$\delta^{13}C$	$\delta^{15}N$	$\delta^{13}C$	$\delta^{15}N$	$\delta^{13}C$	$\delta^{15}N$	$\delta^{13}C$	$\delta^{15}N$
Melissa	-18.9	16.3								
Nigel	-18.1	16.4								
Hang Ten	-17.6	16.5								
Luka	-18.0	16.3								
Easter Egg	-18.6	16.5								
Dicaprio	-17.4	16.5								
Vendredi	-18.4	16.4								
Degas	-18.0	16.6								
Leopard	-17.4	16.5								
Kirby	-17.0	16.2								
Ladwig			-18.0	13.1	-16.2	13.3	-16.9	15.4	-23.1	18.5
BMB	-18.3	15.8	-17.5	15.1			-18.7	17.6		
Skinny Dip			-15.1	16.5	-15.5	16.5	-13.8	18.1	-22.5	11.5
Stefan			-17.1	19.5	-14.9	19.6	-12.8	20.9	-32.9	23.8
Sharkie			-16.8	15.0	-16.5	15.0	-18.5	17.5	-22.5	20.5
Nabby	-19.0	15.4	-17.4	14.3	-17.8	13.9	-19.3	16.2	-23.4	18.0
Carcass							-16.6	19.6		
Icey			-16.2	16.0	-15.4	16.2	-16.9	18.9	-21.9	18.6
Cael			-14.2	19.2	-13.8	19.2	-14.4	20.3		
Pinata			-15.7	17.1			-15.6	19.0	-20.9	25.8
Carcass			-15.9	18.2	-15.3	18.4	-12.5	18.4	-19.6	14.1
Rally	emia		-16.7	16.6	-16.0	16.6	-17.1	18.0	-22.5	22.0
Carcass			-13.2	19.8	-12.7	19.7	-10.8	20.1	-21.6	27.5
Sorenson			-15.5	18.4	-14.9	18.5	-13.6	18.9	-21.0	27.5
Stumpy May	-17.7	12.4	-15.3	16.5	-15.4	16.7	-16.1	18.3	-21.5	15.6
Lord Stanley			-18.5	19.9	-15.3	18.7	-14.9	18.6	-21.5	34.4
Ding			-17.6	18.1	-15.9	18.6	-19.3	19.3		
Shenanigans	-16.3	18.0	-16.3	18.7	-12.4	16.9	-14.3	20.9	-21.4	26.5
Carcass			-16.5	18.7	-16.2	18.8	-14.6	19.7	-23.0	23.8
Sacs			-17.0	18.1	-15.8	18.0	-15.9	19.9	-22.1	20.7
Carcass			-16.6	17.5	-15.4	17.4	-13.8	18.3	-23.4	27.4

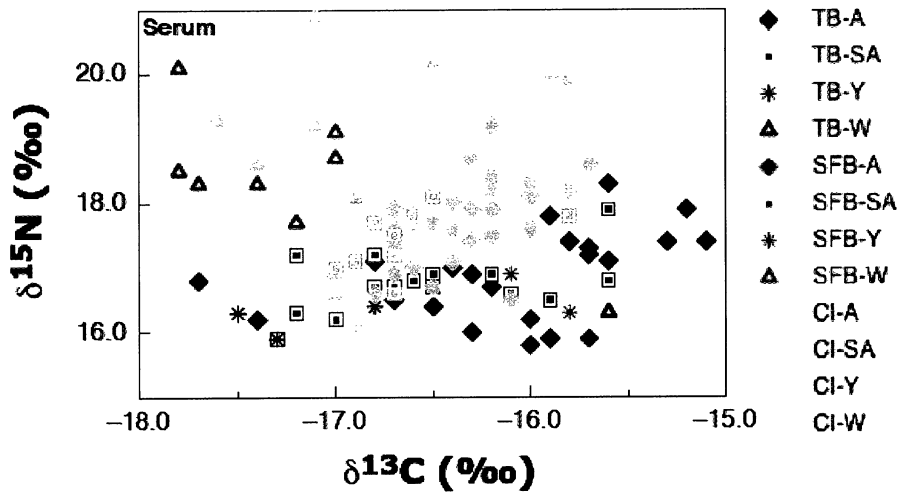
Supplementary Table 1.2: ANOVA statistical analysis of interaction effects between sex, location and age were used for both cell fractions (RBC and serum) on $\delta^{13}C$ and $\delta^{15}N$. Degrees of Freedom (DF), Sum of Squares, F-Ratios, and P-values are given for each test. Significant interactions are those with a $P < 0.05$ (bolded) and are presented in Figures 3 and 4. Both RBC and serum are discussed to determine temporal changes in trophic level feeding behavior in specific groupings.

	RBC $\delta^{13}C$				RBC $\delta^{15}N$			
Source	**DF**	**Sum of Squares**	**F Ratio**	**P value**	**DF**	**Sum of Squares**	**F Ratio**	**P value**
Sex	1	2.25	10.51	<0.01	1	0.34	1.24	0.27
Loc	2	1.40	3.27	0.04	2	3.57	6.44	<0.01
Age	3	0.12	0.19	0.90	3	13.09	15.74	<0.01
Loc*Sex	2	2.14	5.00	**<0.01**	2	4.11	7.42	**<0.01**
Age*Sex	3	0.54	0.85	0.47	3	2.44	2.93	**0.04**
Age*Loc	6	2.31	1.80	0.11	6	1.86	1.12	0.36
Error	82	17.53			82	22.74		
	Serum $\delta^{13}C$				**Serum $\delta^{15}N$**			
Sex	1	1.97	9.33	<0.01	1	0.13	0.37	0.54
Loc	2	0.01	0.02	0.98	2	13.30	18.73	<0.01
Age	3	4.21	6.66	<0.01	3	8.49	7.98	<0.01
Loc*Sex	2	1.96	4.64	**0.01**	2	1.62	2.29	0.11
Age*Sex	3	1.13	1.79	0.16	3	5.23	4.91	**<0.01**
Age*Loc	6	3.98	3.15	**0.01**	6	8.99	4.22	**<0.01**
Error	84	17.71			84	29.82		

Supplementary Figure 1.1: $\delta^{13}C$ and $\delta^{15}N$ equilibration times for serum, muscle (Bulk and Lipid-extracted [LE]), and blubber (Lipid and LE). Each data point represents an *individual* seal, which had either died or was successfully released between days 0 to 93 from admittance date to TMMC (refer to Table S1). Seals were fed salmon oil ($\delta^{13}C$= -21.7‰, $\delta^{15}N$=5.4‰) for the first week, and then switched to ground herring for the remainder of time ($\delta^{13}C$ =-19.4‰, $\delta^{15}N$ =12.5‰).



Supplementary Figure 1.2: Stable carbon *vs* nitrogen in blood serum of harbor seals for all locations. Refer to legend for symbol description. TB = Tomales Bay (black); SFB = San Francisco Bay (dark grey); CI = Channel Islands (light grey); A = Adult > 4 yr (diamond); SA = Subadult 2 - 4 yr (square); Y = Yearling 1 - 2 yr (star); W = Weaner 1 mo- 1 yr (triangle).



APPENDIX 2

Additional tables and figures for Chapter 2

Supplementary Table 2.1: δ^{15}N-AA of captive, rehabilitating harbor seals recovering at TMMC in the Spring of 2007. Sex: Male (M), Female (F); Age: Fetus (F), Pup (P), Adult (A); R.D.E.: Released (R,), Death (D), Euthanized (E). Health status on a scale of 1-7, where 1 is starving and 7 is obese nutritional state according to blubber thickness. Data and TEF calculations within the text is extrapolated from HS serum samples for seals in TMMC greater than two weeks (*i.e.*, equilibrated diet).

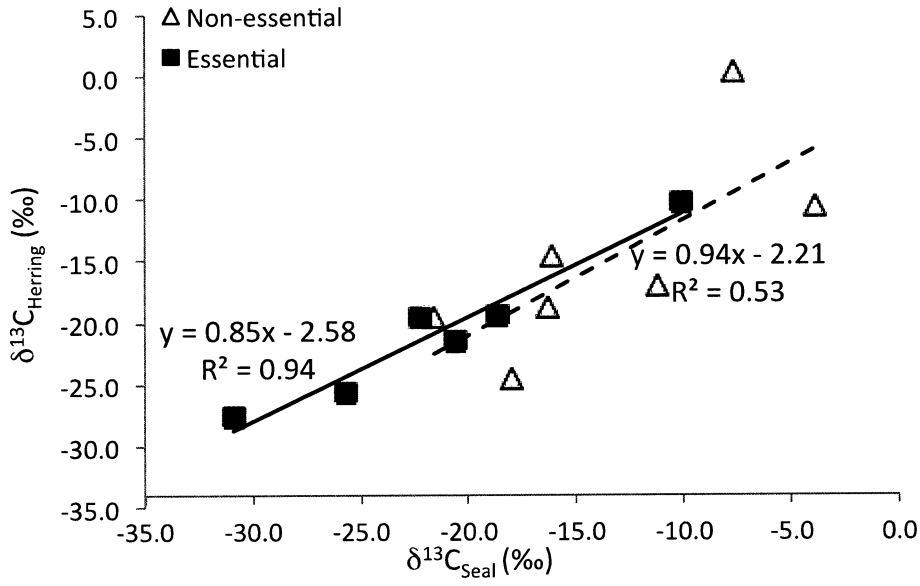
Name	Sample #	Fraction	Sex	Age	R.D.E	Days in Center	Health	Death
BMB	1704	Muscle	F	P	D	36	3	Colitis
Stefan	1707	Muscle	F	A	E	0	2	Septicemica; Pyothroax
Nabby	1718	Muscle	F	P	E	62	6	Neurological
Carcass	1726	Muscle	F	F	D	0	4	Trauma - skull
Carcass	1734	Muscle	F	P	D	0	1	Malnutrition; Fat atrophy
Stumpy May	1739	Muscle	F	P	E	18	3	Aspiration; Enteritis
Shenanigans	1748	Muscle	M	P	E	1	3	Spine Trauma
Carcass	1761	Muscle	?	?	D	0	?	Unknown
Carcass	1719	Blubber	F	A	D	0	4	Bacterial Infection
AA Difference between Serum and Muscle								
Stefan	1707	Corrected Serum	F	A	E	0	2	Septicemica; Pyothroax
Carcass	1726	Corrected Serum	F	F	D	0	4	Trauma - skull
Carcass	1734	Corrected Serum	F	P	D	0	1	Malnutrition; Fat atrophy
Carcass	1761	Corrected Serum	?	?	D	0	?	Unknown

Supplementary Table 2.1 (cont.):

Name	Sample #	Fraction	Ala	Gly	Thr	Ser	Val	Leu	Ile
BMB	1704	Muscle	21.7	20.3	-20.7	14.6	18.1	22.7	21.4
Stefan	1707	Muscle	27.5	20.4	-24.6	19.4	25.0	28.5	28.1
Nabby	1718	Muscle	19.9	18.1	-18.5	13.9	17.6	21.3	19.0
Carcass	1726	Muscle	23.8	18.9	-19.7	16.3	20.9	25.3	25.6
Carcass	1734	Muscle	26.8	21.1	-15.0	17.7	23.6	28.1	25.1
Stumpy May	1739	Muscle	23.3	19.4	-19.9	13.3	18.6	23.6	24.3
Shenanigans	1748	Muscle	25.5	22.7	-26.9	16.4	21.8	26.2	25.4
Carcass	1761	Muscle	22.4	21.2	-25.6	17.0	21.9	24.8	21.5
Carcass	1719	Blubber	27.2	22.9	-22.5	17.0	20.1	25.0	24.8
AA Difference between Serum and Muscle			.2 ± 1.2	-7.8 ± 0.2	-1.1 ± 1.4	-4.1 ± 0.3	3.0 ± 2.0	1.0 ± 0.7	0.2 ± 1.5
Stefan	1707	Corrected Serum	28.8	12.6	-25.7	15.3	28.1	29.5	28.3
Carcass	1726	Corrected Serum	25.1	11.1	-20.8	12.3	23.9	26.2	25.8
Carcass	1734	Corrected Serum	28.1	13.3	-16.1	13.7	26.6	29.1	25.3
Carcass	1761	Corrected Serum	23.6	13.5	-26.7	13.0	25.0	25.8	21.8

Name	Sample #	Fraction	Pro	Asp	Glu	Phe	Lys	Tyr	Arg	Bulk
BMB	1704	Muscle	23.7	19.3	23.9	11.0	6.2	14.8	7.0	
Stefan	1707	Muscle	26.8	24.3	29.9	20.1	13.9	18.8	17.2	19.6
Nabby	1718	Muscle	21.8	17.4	22.5	11.5	6.6	13.8	7.9	13.9
Carcass	1726	Muscle	26.3	22.5	27.9	15.7	12.2	11.8		18.4
Carcass	1734	Muscle	28.4	24.5	28.8	19.4	10.6	17.7		19.7
Stumpy May	1739	Muscle	27.6	20.7	24.5	15.4	5.8	13.1		16.7
Shenanigans	1748	Muscle	30.3	22.2	27.6	16.1	12.8	17.1	17.0	16.9
Carcass	1761	Muscle	27.4	21.7	26.4	16.6	7.2	15.4		17.4
Carcass	1719	Blubber	28.0	22.3	26.0	11.4		22.3	12.2	19.6
AA Difference between Serum and Muscle			5.0 ± 0.0	-0.2 ± 2.2	0.4 ± 2.1	-1.4 ± 1.4	-0.1 ± 0.8	5.1 ± 0.9		
Stefan	1707	Corrected Serum	31.8	24.1	30.3	18.8	13.8	23.9		
Carcass	1726	Corrected Serum	31.3	22.2	28.3	14.4	12.1	16.9		
Carcass	1734	Corrected Serum	33.4	24.2	29.2	18.1	10.5	22.8		
Carcass	1761	Corrected Serum	32.5	21.4	26.8	15.3	7.1	20.5		

Supplementary Figure 2.1: Linear regressions of $\delta^{13}C_{Seal}$ vs. $\delta^{13}C_{Herring}$ for essential and non-essential AA groupings. Essential AA (squares) are derived from prey, and thus have a high R^2. Non-essential AA (triangles) are biosynthesized within the tissue and have a low R^2.



Supplementary Methods Text 2:

2.1 More detailed methodology and instrument settings for $\delta^{15}N$ and $\delta^{13}C$ compound-specific amino acid measurements

Typical analytical protocol: 1 mg of dry sample was hydrolyzed (6N hydrochloric acid for 19 hr at 110°C), dried under a N_2 stream, and desiccated overnight. Individual amino acids were subsequently derivatized to triflouroacetyl/isopropyl esters, using a modified protocol of Silfer et al. (1991). First a mixture of 5:1 (vol:vol) isopropyl:acetic chloride was added, then samples were heated to 110°C for 1 hr, and again dried under N_2. Next a mixture of 3:2 (vol: vol) dichloromethane:trifluoroacetic acid anhydride was added, samples were heated to 100°C for 15 min, cooled, and gently evaporated just to dryness under N_2. Samples were finally brought up in dichloromethane for GC-MS analysis. Individual amino acid isotope ratios were quantified via the GC-C-IRMS (Thermo Trace Ultra GC fitted with a Agilent DB-5 column; 50 m x 0.32 mm ID x 0.52 μm film thickness), in line with an oxidation furnace (set at 980°C for N and 940°C for C) and a reduction furnace (set at 650°C for N and 630°C for C), linked to a Finnagin Delta^{Plus} XP mass spectrometer. GC conditions were as follows for N: 1 μl splitless injection, 2 ml/min He in constant-flow mode, injector temp. 250°C. Oven program settings were as follows: initial temp. = 52°C, hold for 2 min; ramp 1 = 15°C/min to 75°C, hold for 2 min; ramp 2 = 4°C/min to 185°C, hold for 2 min; ramp 3 = 4°C/min to 200°C; ramp 4 = 30°C/min to 240°C, hold for 5 min. GC conditions were as follows for C: 1 μl split (5:1) injection, 2.1 ml/min He in constant-flow mode, injector temp. 250°C. Oven program settings were as follows: initial temp. = 35°C, hold for 2 min; ramp 1 = 15°C/min to 75°C, hold for 2 min; ramp 2 = 4°C/min to 90°C, hold for 2 min; ramp 3 = 4°C/min to 200°C; ramp 4 = 30°C/min to 240°C, hold for 5 min.

Each derivatized sample was injected in quadruplicate and isotope values averaged. The average precision (analytical error) associated with quadruplicate

injections of samples was ~0.8‰. To ensure accuracy of $\delta^{15}N$ values, all samples were derivatized with two accompanying external standards: 1) a 'working' amino acid standard, and 2) cyanobacteria, where $\delta^{15}N$ values were determined independently via an elemental analyzer-IRMS. This 'working standard' was injected before and after each group of sample injections to verify the stability of $\delta^{15}N$ values throughout each GC-C-IRMS analysis. In addition, an internal standard (nor-Leu) with a known $\delta^{15}N$ value was added to each sample to ensure the integrity of individual sample handling and derivatization. In the 'working' standard, over various runs, the average standard deviation of $\delta^{15}N$ values for all amino acids was 1.1‰, where specific amino acids standard deviations were as follows: Glu, 0.9‰; Phe, 0.6 ‰, and nor-Leu, 0.5‰. For $\delta^{13}C$ measurements, values were corrected based on results from an external standard using the method of Silfer et al. (1991), which accounts for both carbon added and also fractionation during derivatization.

2.2 More Detailed derivation of multi-TEF equation to calculate trophic position (TP)

Using the approach after Pauly et al. (1998), the final TP for seals can be expressed as the sum of TP for each of these stages:

(5) $TP_{Seal} = [(\Delta^{15}N_{Glu\text{-}Phe,Seal} - \Delta^{15}N_{Glu\text{-}Phe,Fish} / TEF_{Glu\text{-}Phe,Urea}) + (\Delta^{15}N_{Glu\text{-}Phe,Fish} - 3.4 / TEF_{Glu\text{-}Phe,Ammonia}]) + 1,$

where $\Delta^{15}N_{Glu\text{-}Phe,Seal}$ and $\Delta^{15}N_{Glu\text{-}Phe,Fish} = \delta^{15}N_{Glu} - \delta^{15}N_{Phe}$ measured in the seal and fish, respectively, 3.4 is $\delta^{15}N_{Glu} - \delta^{15}N_{Phe}$ in primary producers, $TEF_{Urea} = 4.3‰$ and $TEF_{Ammonia} = 7.6‰$ (McClelland and Montoya 2002) and 1 is added to represent the first trophic position occupied by primary producers at the base of the food web plankton. If we assume that the transfer from seal to fish represents 1 TP, then

(6) $\Delta^{15}N_{Glu\text{-}Phe,Seal} - \Delta^{15}N_{Glu\text{-}Phe,Fish} / TEF_{Glu\text{-}Phe,Urea} = 1$,

and after substitution into equation (5)

(7) $TP_{Seal} = (\Delta^{15}N_{Glu\text{-}Phe,Fish} - 3.4 / TEF_{Glu\text{-}Phe,Ammonia}) + 2$.

For wild animals we might only be able to measure isotopic values in seals, so the $\Delta_{Glu\text{-}Phe,Fish}$ might not determined. Based on the definition of TEF_{Urea}, and following rearrangement:

(8) $\Delta^{15}N_{Glu\text{-}Phe,Fish} = \Delta^{15}N_{Glu\text{-}Phe,Seal} - TEF_{Urea}$.

Finally, with substitution into equation (7) a final equation based only on measured Phe and Glu $\delta^{15}N$ values in seals, becomes:

(9) $TP_{Seal} = [(\Delta^{15}N_{Glu\text{-}Phe,Seal} - TEF_{Glu\text{-}Phe,Urea} - 3.4) / TEF_{Glu\text{-}Phe,Ammonia}]) + 2$,

or, after substitution:

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

APPENDIX 3

Additional tables and figures for Chapter 3

Supplementary Table 3.1: δ^{13}C-AA of harbor seals off the California coast in the Spring of 2007. δ^{13}C-AA values and standard deviations of wild harbor seals in both serum and red blood cell (RBC) fractions. Each AA was injected three times. GC-IRMS Injection Date and Filename ID # included. Male (M), Female (F); Age: Adult (A), Subadult (SA), Yearling (Y), Weaner (W); Location (Loc): Channel Islands (CI), San Francisco Bay (SFB), Tomales Bay (TB).

Sample ID #	Blood Fraction	GC-IRMS Date	GC-IRMS Filename ID	Sex	Age	Loc
1546	RBC	12/07/07	GC_C_071207_LR WHS RBC and JL UDOM extraction-0409.cf	M	A	CI
1502	RBC	12/08/07	GC_C_071207_LR WHS RBC and JL UDOM extraction-0421.cf	M	Y	CI
1458/59	RBC	12/04/07	GC_C_071204_LR_WHSRBC 1458-0396.cf	F	A	SFB
1580/81	RBC	12/08/07	GC_C_071207_LR WHS RBC and JL UDOM extraction-0417.cf	M	Y	SFB
1547/51	RBC	12/07/07	GC_C_071207_LR WHS RBC and JL UDOM extraction-0405.cf	M	W	SFB
1546	Serum	03/06/08	GC_C_LR_080306_WHS1546.1502.1542.cyano-0718.cf	M	A	CI
1503	Serum	04/04/08	GC_C_LR_080404_WHS1503.1517.1519.1574.1576.1578.cyano-0771.	M	SA	CI
1517	Serum	01/14/09	GC_C_090114_LR_WHS1517.1519-1765.cf	F	SA	CI
1498	Serum	01/13/09	GC_C_090113_LR_WHS1492.1498-1753.cf	F	Y	CI
1502	Serum	03/06/08	GC_C_LR_080306_WHS1546.1502.1542.cyano-0721.cf	M	Y	CI
1489	Serum	01/12/09	GC_C_090112_LR_WHS1456.1489.1490-1741.cf	F	W	CI
1492	Serum	04/04/08	GC_C_LR_080404_WHS1492.1498-0764.cf	M	W	CI
1490	Serum	01/12/09	GC_C_090112_LR_WHS1456.1489.1490-1745.cf	F	W	CI
1542/43	Serum	03/07/08	GC_C_LR_080307_WHS1542-0734.cf	F	W	CI
1578/79	Serum	01/15/09	GC_C_090115_LR_WHS1578.1576-1787.cf	F	A	SFB
1458/59	Serum	03/05/08	GC_C_LR_080305_WHS1547.1458.1580-0710.cf	F	A	SFB
1460/61	Serum	04/03/08	GC_C_LR_080403_WHS1456.1460.1489.1490-0752.cf	F	A	SFB
1445/1588	Serum	04/02/08	GC_C_LR_080402_AA1testWHS1445-0741.cf	M	SA	SFB
1456/57	Serum	01/13/09	GC_C_090113_LR_WHS1456.1517.1519.1576-1757.cf	F	SA	SFB
1598/99	Serum	01/16/09	GC_C_090116_LR_WHS 1592.94.96.98.1657.cyano2-1815.cf	F	SA	SFB
1580/81	Serum	03/06/08	GC_C_LR_080305_WHS1547.1458.1580-0714.cf	M	Y	SFB
1590/91	Serum	01/16/09	GC_C_090116_LR_WHS1586.1590 after 5 min oxid-1800.cf	M	Y	SFB
1592/93	Serum	01/16/09	GC_C_090116_LR_WHS 1592.94.96.98.1657.cyano2-1803.cf	F	Y	SFB
1519/20	Serum	01/14/09	GC_C_090114_LR_WHS1517.1519-1769.cf	M	W	SFB
1574/75	Serum	04/06/08	GC_C_LR_080406_WHS1517.1576.1574-0808.cf	F	W	SFB
1594/95	Serum	01/16/09	GC_C_090116_LR_WHS 1592.94.96.98.1657.cyano2-1807.cf	M	W	SFB
1596/97	Serum	01/16/09	GC_C_090116_LR_WHS 1592.94.96.98.1657.cyano2-1811.cf	F	W	SFB
1547/51	Serum	03/05/08	GC_C_LR_080305_AAIsoQ-A-Der1_1-0707.cf	M	W	SFB
1576/77	Serum	01/15/09	GC_C_090115_LR_WHS1578.1576-1791.cf	F	W	SFB
1450/51	Serum	04/02/08	GC_C_LR_080403_WHS1445.1450-0745.cf	F	Y	TB
1657/58	Serum	01/16/09	GC_C_090116_LR_WHS 1592.94.96.98.1657.cyano2-1819.cf	F	W	TB
CARBON ONLY						
1542/43	RBC	12/07/07	GC_C_071207_LR WHS RBC and JL UDOM extraction-0413.cf	F	W	CI
1584/85	RBC	08/24/07	GC_C_CyanoBSAWHS1584_23Aug07-0010.cf	M	A	SFB
1659/60	RBC	08/26/07	GC_C_070826_LAR_WHSBloodRun-0215.cf	F	A	TB
1584/85	Serum	08/26/07	GC_C_070826_LAR_WHSBloodRun-0211.cf	M	A	SFB
1586/87	Serum	01/16/09	GC_C_090116_LR_WHS1586.1590 after 5 min oxid-1796.cf	M	W	SFB
1659/60	Serum	08/26/07	GC_C_070826_LAR_WHSBloodRun-0219.cf	F	A	TB

Supplementary Table 3.1 (cont.):

		$\delta^{13}C$ (‰)													
Sample ID #	Blood Fraction	Ala	S.D.	Gly	S.D.	Thr	S.D.	Ser	S.D.	Val	S.D.	Leu	S.D.	Ile	S.D.
1546	RBC	-19.4	0.8	0.1	0.5	-6.9	0.4	3.2	0.3	-20.4	0.5	-17.9	0.7	10.0	0.7
1502	RBC	-10.2	0.6	-6.3	0.0	-6.3	0.3	-1.0	1.0	-23.1	0.5	-19.2	0.1	-29.7	0.3
1458/59	RBC	-12.8	1.0	-2.3	1.9	-5.3	0.8	-1.1	0.4	-18.7	0.4	-23.6	0.5	-14.5	1.1
1580/81	RBC	-18.7	0.4	-0.2	0.4	-7.4	0.1	6.4	0.3	-22.2	0.0	-20.5	0.1	18.8	0.0
1547/51	RBC	-16.3	0.4	-4.3	0.5	0.7	0.6	-0.1	0.2	-17.7	0.7	-16.7	0.2	11.7	0.7
1546	Serum	-17.0	0.6	1.8	1.1	-2.8	0.6	1.4	0.7	-14.4	0.5	-23.0	0.3	-9.6	2.5
1503	Serum	-16.5	0.4	-17.5	0.6	-1.4	0.8	1.0	0.9	-22.1	1.2	-25.8	0.9	-19.0	1.1
1517	Serum	-11.0	0.5	-0.2	0.7	-3.2	0.2	-8.5	0.5	-17.9	0.4	-23.3	0.1	-13.5	0.2
1498	Serum	-14.6	0.9	0.6	0.5	-7.3	0.3	1.2	0.5	-16.6	0.6	-24.9	0.2	-17.1	0.6
1502	Serum	-16.2	0.5	-10.7	0.7	-3.9	0.3	-10.1	0.2	-16.1	0.1	-23.7	0.1	-12.7	0.9
1489	Serum	-14.7	0.3	0.4	0.7	-2.5	0.1	6.3	0.4	-14.5	0.1	-23.2	0.4	-14.3	1.9
1492	Serum	-21.0	1.4	25.5	1.3	-4.3	1.1	-16.7	0.8	-21.8	0.5	-19.1	0.2	-28.8	9.6
1490	Serum	-20.1	0.7	-3.3	0.7	-8.9	0.9	3.8	0.5	-13.9	0.4	-23.5	0.2	-15.6	0.1
1542/43	Serum	-20.4	0.6	-5.8	0.7	-3.5	0.9	-9.5	0.6	-13.0	0.4	-21.3	0.2	-9.1	1.7
1578/79	Serum	-12.7	0.5	4.6	1.0	-1.5	0.5	-1.1	0.3	-15.4	0.2	-24.1	0.3	-15.2	1.0
1458/59	Serum	-17.2	0.5	-7.4	0.8	-7.1	0.2	-9.7	0.2	-16.0	0.1	-22.4	0.2	-11.8	0.8
1460/61	Serum	-14.4	0.3	2.0	0.7	-2.3	0.0	1.0	0.3	-14.3	0.1	-20.8	0.3	-24.6	6.4
1445/1588	Serum	-13.5	1.7	6.5	0.3	-8.3	0.3	-9.2	0.5	-15.7	0.6	-22.4	2.2	-16.6	0.8
1456/57	Serum	-17.0	0.2	6.1	0.2	-1.1	0.6	-0.3	0.4	-19.6	0.5	-23.8	0.3	-15.6	0.1
1598/99	Serum	-14.2	0.3	4.7	0.3	-5.1	0.0	-3.8	0.2	-21.0	0.1	-24.0	0.5	-21.6	0.4
1580/81	Serum	-17.1	1.1	6.1	0.5	-2.1	0.4	-4.4	0.3	-16.3	0.2	-24.2	0.3	-12.0	0.8
1590/91	Serum	-20.3	0.5	-7.5	0.6	-12.5	0.4	-7.7	0.2	-25.5	0.7	-28.5	0.3	-34.7	0.0
1592/93	Serum	-17.5	0.3	-4.4	0.3	-3.9	0.1	0.0	0.1	-18.5	0.1	-23.5	0.3	-17.9	0.3
1519/20	Serum	-18.6	0.3	13.1	0.6	-3.8	0.4	1.6	0.2	-15.7	0.1	-27.0	0.3	-19.3	1.9
1574/75	Serum	-13.4	0.5			-4.4	0.1	4.7	0.2	-7.3	0.6	-21.2	1.5	-9.1	0.2
1594/95	Serum	-22.9	0.1	0.2	0.4	-4.0	0.2	3.7	0.2	-19.1	0.4	-24.6	0.2	-18.9	0.2
1596/97	Serum	-16.4	0.2	2.6	0.1	0.8	0.3	13.0	0.2	-12.7	0.4	-19.4	0.5	-10.7	0.6
1547/51	Serum	-12.8	1.2	-4.5	3.4	-3.3	1.6	2.4	0.3	-16.5	0.2	-25.8	1.3	-10.4	1.0
1576/77	Serum	-17.4	0.1	0.0	0.1	-12.4	0.1	-2.4	0.1	-11.8	0.4	-23.0	0.2	-16.0	0.9
1450/51	Serum	-8.7	1.8	15.1	1.1	-6.4	0.9	-11.3	1.1	-21.6	1.7	-25.6	0.7	-31.8	0.2
1657/58	Serum	-12.8	0.1	5.0	0.6	3.7	0.5	4.0	0.3	-15.9	1.0	-21.8	0.8	-14.3	0.1
CARBON ONLY															
1542/43	RBC	-18.4	0.2	5.2	0.8	-5.2	0.4	13.1	0.1	-24.9	0.2	-23.9	0.1	18.9	0.4
1584/85	RBC	-18.1	0.4	-3.4	0.2	-7.7	0.3	-1.7	0.6	-20.0	0.2	-25.8	0.2	-26.4	1.2
1659/60	RBC	-15.8	0.2	-2.1	0.1	-8.1	0.3	-7.0	0.3	-18.8	0.4	-26.5	0.3	1.6	0.5
1584/85	Serum	-17.3	0.3	-5.4	0.2	-7.6	0.3	-9.2	0.2	-19.7	0.8	-24.5	0.1	-12.7	1.0
1586/87	Serum	-13.9	0.1	5.4	0.5	-1.5	0.0	2.6	0.1	-17.7	1.0	-26.8	0.9	-14.0	0.7
1659/60	Serum	-14.7	0.1	-4.4	0.1	-6.5	0.2	-7.3	0.2	-18.6	0.3	-26.6	0.2	-10.6	0.2

Supplementary Table 3.1 (cont.):

Sample ID #	Blood Fraction	Pro	S.D.	Asp	S.D.	Glu	S.D.	Phe	S.D.	Lys	S.D.	Tyr	S.D.	Arg	S.D.
1546	RBC	-15.8	0.5	-11.3	0.3	-11.4	0.4	-22.0	0.1	-16.4	0.3	-23.8	0.1	-22.3	0.8
1502	RBC	-14.6	0.1	-16.6	0.7	-16.0	0.1	-23.3	0.3	-17.5	0.4	-24.1	0.4	-21.6	0.3
1458/59	RBC	-16.5	0.5	-15.1	0.4	-13.8	0.2	-23.2	0.1	-15.9	1.1	-22.1	0.4		
1580/81	RBC	-16.2	0.2	-17.0	0.2	-14.4	0.1	-23.9	0.4	-17.1	0.5	-23.3	0.1	-22.5	0.3
1547/51	RBC	-15.5	0.3	-10.9	0.3	-11.3	0.3	-22.9	0.1	-17.9	0.2	-24.4	0.1	-20.0	0.2
1546	Serum	-11.2	0.8	-7.1	1.0	-11.0	0.4	-27.0	0.3	-17.1	0.3	-24.6	1.9	-15.0	0.9
1503	Serum	-16.8	0.8	-14.0	0.4	-11.9	0.2	-26.7	1.0	-19.4	0.3	-32.8	0.5	-15.6	0.4
1517	Serum	-12.7	1.7	-12.1	1.0	-16.3	0.6	-26.1	1.1	-17.1	0.8	-21.9	0.4	-13.3	0.6
1498	Serum	-17.0	0.4	-5.8	0.4	-15.4	0.3	-26.1	0.2	-18.6	0.6	-22.7	0.9		
1502	Serum	-15.2	0.4	-9.7	0.0	-12.4	0.2	-25.7	0.1	-16.0	0.4	-21.0	0.4	-21.3	2.0
1489	Serum	-14.1	0.2	-7.9	0.4	-13.6	0.3	-24.6	0.4	-17.0	0.3	-23.5	0.4	-4.3	
1492	Serum	-4.8	0.4	-12.4	1.1	-13.6	0.2	-29.1	0.1	-16.8	0.7	-30.1	0.4		
1490	Serum	-13.1	0.8	-13.2	1.0	-13.6	0.3	-24.1	0.5	-16.4	0.2	-20.2	1.5	-13.6	
1542/43	Serum	-13.8	0.5	-10.3	0.9	-12.3	0.7	-25.6	0.3	-15.8	0.6	-19.8	1.1		
1578/79	Serum	-9.3	0.6	-6.1	0.8	-6.2	0.6	-23.3	0.3	-14.9	0.6	-23.6	0.1		
1458/59	Serum	-13.7	0.1	-9.6	0.1	-12.2	0.2	-22.3	0.2	-16.8	0.3	-27.6	0.3	-16.4	0.2
1460/61	Serum	-17.3	0.7	-15.5	0.2	-6.7	0.4	-20.6	0.7	-12.3	1.5				
1445/1588	Serum	-12.0	0.9	-6.7	0.0	-6.4	0.5	-29.6	0.4	-19.4	0.3	-26.7	0.6	-20.9	0.6
1456/57	Serum	-14.2	0.4	-5.6	0.5	-11.4	0.6	-26.1	0.3	-16.4	0.2				
1598/99	Serum	-11.2	0.2	-16.2	0.0	-11.7	0.1	-26.4	0.4	-15.6	0.3	-24.6	0.3		
1580/81	Serum	-14.8	0.3	-11.0	0.1	-12.2	0.2	-25.1	0.6	-16.6	0.3	-24.6	1.1	-14.5	0.9
1590/91	Serum	-19.2	0.7	-18.3	0.4	-16.7	0.3	-26.8	0.1	-16.6	0.1	-25.0	0.2	-18.9	0.5
1592/93	Serum	-14.1	0.5	-13.8	0.3	-12.8	0.1	-22.2	0.1	-16.2	0.2	-22.6	0.2	-18.2	0.1
1519/20	Serum	-13.5	0.9	-10.4	0.2	-16.5	0.2	-27.2	0.1	-17.2	0.1	-15.8	0.9		
1574/75	Serum	-13.3	0.8	-19.5	0.8	-13.3	0.3	-22.0	0.2	-15.9	0.1	-21.8	0.4	-15.6	0.3
1594/95	Serum	-18.2	0.7	-16.2	0.4	-14.9	0.5	-23.6	0.4	-18.9	0.5	-24.5	0.3	-19.0	0.1
1596/97	Serum	-8.3	0.6	-14.3	0.0	-12.8	0.2	-23.3	0.3	-12.7	0.4	-21.9	0.2	-13.2	0.0
1547/51	Serum	-13.6	1.1	-15.5	0.6	-17.4	0.4	-26.0	0.8	-19.1	0.1	-26.3	0.7	-18.4	0.1
1576/77	Serum	-6.0	0.3	-11.0	0.3	-13.0	0.2	-22.7	0.0	-14.3	0.3	-22.5	0.3		
1450/51	Serum	-19.4	1.6	-2.4	1.6	-3.2	0.4	-34.1	1.5	-18.1	0.5	-24.5	2.8	-19.1	0.8
1657/58	Serum	-10.5	0.1	-11.0	0.7	-11.7	0.6	-25.1	0.4	-14.4	0.0	-22.8	0.3	-12.0	0.1
CARBON ONLY															
1542/43	RBC	-19.5	0.5	-15.6	0.4	-9.8	0.2	-22.1	0.2	-15.7	0.3	-23.1	0.2		
1584/85	RBC	-15.9	0.2	-17.3	0.2	-13.7	0.1	-23.3	0.2	-11.4	0.3	-23.2	0.0		
1659/60	RBC	-14.7	0.3	-18.4	0.1	-15.1	0.1	-23.4	0.2	-13.7	0.2	-22.7	0.2		
1584/85	Serum	-14.6	0.3	-16.1	0.1	-16.2	0.4	-24.5	0.1	-15.4	0.4	-22.8	0.3		
1586/87	Serum	-11.9	0.6	-19.2	0.3	-20.9	0.1	-24.4	0.3	-18.4	0.7	-22.9	0.6		
1659/60	Serum	-13.5	0.2	-15.8	0.2	-15.3	0.4	-24.1	0.3	-15.2	0.4	-22.6	0.5		

Supplementary Table 3.2: δ^{15}N-AA of harbor seals off the California coast in the Spring of 2007. δ^{15}N-AA values and standard deviations of wild harbor seals in both serum and red blood cell (RBC) fractions. Each AA was injected four times. GC-IRMS Injection Date and Filename ID # included. Sex: Male (M), Female (F); Age: Adult (A), Subadult (SA), Yearling (Y), Weaner (W); Location (Loc): Channel Islands (CI), San Francisco Bay (SFB), Tomales Bay (TB). Trophic position was calculated using equation (2) in methods.

Sample ID #	Blood Fraction	GC-IRMS Date	GC-IRMS Filename ID	Sex	Age	Loc
1546	RBC	12/12/07	GC_N_071212_LR_WHS RBC-0487.cf	M	A	CI
1502	RBC	12/11/07	GC_N_071211_LR WHS RBC-0470.cf	M	Y	CI
1458/59	RBC	12/12/07	GC_N_071212_LR_WHS RBC-0484.cf	F	A	SFB
1580/81	RBC	12/11/07	GC_N_071211_LR WHS RBC-0465.cf	M	Y	SFB
1547/51	RBC	12/11/07	GC_N_071211_LR WHS RBC-0460.cf	M	W	SFB
1546	Serum	03/01/08	GC_N_LR_080301_WHS 1547.1546-S-0683.cf	M	A	CI
1503	Serum	04/13/08	GC_N_LR_080413_WHS1503.1519-0882.cf	M	SA	CI
1517	Serum	01/21/09	GC_N_090121_LR_WHS1517-1850.cf	F	SA	CI
1498	Serum	01/20/09	GC_N_090120_LR_WHS1456.1489.1490.1498-1845.cf	F	Y	CI
1502	Serum	03/02/08	GC_N_LR_080302_WHS 1542S.1502S.cyano-0693.cf	M	Y	CI
1489	Serum	01/20/09	GC_N_090120_LR_WHS1456.1489.1490.1498-1835.cf	F	W	CI
1492	Serum	04/12/08	GC_N_LR_080412_WHS1490.1492.1498-0872.cf	M	W	CI
1490	Serum	01/20/09	GC_N_090120_LR_WHS1456.1489.1490.1498-1840.cf	F	W	CI
1542/43	Serum	03/02/08	GC_N_LR_080302_WHS 1542S.1502S.cyano-0688.cf	F	W	CI
1578/79	Serum	01/21/09	GC_N_090121_LR_WHS1519.1576.1578.cyano-1870.cf	F	A	SFB
1458/59	Serum	03/01/08	GC_N_LR_080230_AAIsoQDer-A-1-1-0669.cf	F	A	SFB
1460/61	Serum	04/11/08	GC_N_LR_080411_WHS1445.1450.1456.1460-0854.cf	F	A	SFB
1445/1588	Serum	04/11/08	GC_N_LR_080411_WHS1445.1450.1456.1460-0838.cf	M	SA	SFB
1456/57	Serum	01/20/09	GC_N_090120_LR_WHS1456.1489.1490.1498-1830.cf	F	SA	SFB
1598/99	Serum	01/23/09	GC_N_090123_LR_WHS 1592.1594.1596.1598-1908.cf	F	SA	SFB
1580/81	Serum	03/01/08	GC_N_LR_080301_WHS 1458.1580-S-0674.cf	M	Y	SFB
1590/91	Serum	01/23/09	GC_N_090123_LR_AA3 and WHS1590-1888.cf	M	Y	SFB
1592/93	Serum	01/23/09	GC_N_090123_LR_WHS 1592.1594.1596.1598-1893.cf	F	Y	SFB
1519/20	Serum	01/21/09	GC_N_090121_LR_WHS1519.1576.1578.cyano-1860.cf	M	W	SFB
1574/75	Serum	04/15/08	GC_N_LR_080413_WHS1576.1574.cyano.1578-0896.cf	F	W	SFB
1594/95	Serum	01/23/09	GC_N_090123_LR_WHS 1592.1594.1596.1598-1898.cf	M	W	SFB
1596/97	Serum	01/23/09	GC_N_090123_LR_WHS 1592.1594.1596.1598-1903.cf	F	W	SFB
1547/51	Serum	03/02/08	GC_N_LR_080301_WHS 1547.1546-S-0678.cf	M	W	SFB
1576/77	Serum	01/21/09	GC_N_090121_LR_WHS1519.1576.1578.cyano-1865.cf	F	W	SFB
1450/51	Serum	04/11/08	GC_N_LR_080411_WHS1445.1450.1456.1460-0843.cf	F	Y	TB
1657/58	Serum	01/26/09	GC_N_090126_LR_WHS 1657.cyano2-1913.cf	F	W	TB

Supplementary Table 3.2 (cont.):

		$\delta^{15}N$ (‰)													
Sample ID #	Blood Fraction	Ala	S.D.	Gly	S.D.	Thr	S.D.	Ser	S.D.	Val	S.D.	Leu	S.D.	Ile	S.D.
1546	RBC	25.1		16.9		-21.2		18.0		26.3		27.1		18.7	
1502	RBC	24.1	0.4	13.9	1.3	-16.8	0.8	14.6	1.8	25.0	1.5	25.1	1.0	24.4	3.7
1458/59	RBC	22.7	1.0	12.4	1.1	-14.9	1.9	12.2	6.0	21.5	2.1	24.3	0.5	25.4	
1580/81	RBC	22.6	1.4	12.5	0.9	-16.4	1.5	12.6	0.1	25.9	0.9	25.5	0.4	27.8	2.7
1547/51	RBC	25.3	0.7	15.5	0.7	-23.4	0.3	18.6	0.7	27.4	1.6	28.8	1.6	25.1	2.0
1546	Serum	27.7	1.0	14.3	0.4	-26.8	1.4	17.5	0.4	29.5	1.0	27.5	0.5	30.4	1.2
1503	Serum	14.9	1.0	12.4	2.0	-13.9	1.1	11.7	1.0	20.8	0.8	24.1	1.4	23.6	0.8
1517	Serum	21.9	1.6	13.8	0.8	-16.4	2.8	13.4	2.4	21.8	0.7	25.7	0.3	25.8	4.5
1498	Serum	22.1	1.0	12.8	0.3	-19.9	0.9	11.8	1.6	21.3	0.4	24.6	0.6	25.0	1.2
1502	Serum	21.2	0.6	10.6	0.9	-18.0	0.8	11.8	0.6	24.1	0.9	22.3	0.4	22.4	0.6
1489	Serum	25.9	0.6	16.9	0.7	-25.0	0.9	12.2	0.8	21.0	1.2	26.3	0.5	23.1	1.3
1492	Serum	21.4	2.3	15.5	6.3	-14.9	3.2	17.7	0.6	20.5	2.1	23.4	0.3	24.3	0.4
1490	Serum	25.0	0.6	16.7	0.5	-26.4	0.9	14.5	1.3	23.2	0.9	27.7	0.9	30.0	1.9
1542/43	Serum	25.1	0.7	11.1	1.9	-27.7	1.8	15.5	0.8	26.0	0.5	26.4	0.6	26.8	3.7
1578/79	Serum	22.0	0.8	11.5	0.0	-15.5	1.1	12.5	0.4	18.0	1.0	23.0	0.9	17.9	1.0
1458/59	Serum	21.9	2.0	10.7	0.3	-16.5	0.5	12.7	1.9	25.9	2.0	24.8	0.2	25.5	1.2
1460/61	Serum	20.9	0.5	12.4	1.0	-11.2	1.0	8.4	0.4	17.8	0.7	20.6	0.8	18.2	1.9
1445/1588	Serum	20.5	0.9	16.0	1.7	-14.6	0.8	14.7	1.3	22.7	1.4	22.7	1.5	20.2	0.3
1456/57	Serum	22.3	0.7	14.4	1.0	-22.9	0.6	11.8	0.6	19.6	1.5	22.5	0.5	22.4	0.8
1598/99	Serum	22.4	0.5	15.8	1.0	-16.5	0.4	12.2	1.3	13.1	0.7	23.0	0.8	19.0	1.0
1580/81	Serum	23.8	0.5	11.7	0.3	-20.0	0.3	14.6	0.8	25.4	0.8	23.5	0.7	23.8	1.7
1590/91	Serum	22.9	1.0	12.9	0.6	-18.4	1.4	10.6	0.9	16.2	0.5	23.0	0.3	21.3	2.4
1592/93	Serum	21.5	0.3	13.8	0.7	-13.2	1.4	12.4	0.5	17.0	1.8	24.2	0.7	25.0	1.5
1519/20	Serum	20.4	0.6	9.3	0.7	-20.9	0.9	15.5	0.5	19.8	1.2	22.5	1.1	21.6	0.6
1574/75	Serum	9.8	4.1			-13.2	0.8	5.4	0.5	8.9	0.7	19.2	3.8	12.4	1.5
1594/95	Serum	22.5	1.3	16.5	0.8	-16.2	1.0	12.8	2.1	17.9	0.9	23.6	0.4	23.1	1.0
1596/97	Serum	23.4	0.8	17.4	0.9	-16.2	3.0	8.9	1.2	17.5	0.1	27.0	0.4	24.1	1.8
1547/51	Serum	26.8	1.1	12.4	2.3	-23.0	0.4	19.5	2.0	28.3	0.9	26.7	0.5	27.3	1.0
1576/77	Serum	23.8	0.5	14.2	0.4	-19.0	0.9	13.7	1.0	19.3	1.0	25.6	0.2	30.4	1.9
1450/51	Serum	20.1	1.1	15.3	1.6	-19.0	0.7	13.9	1.9	23.9	0.9	22.1	1.2	19.5	0.4
1657/58	Serum	21.0	0.6	14.8	0.7	-11.3	0.6	9.7	1.0	17.0	1.9	23.4	0.8	25.6	1.2

Supplementary Table 3.2 (cont.):

Sample ID #	Blood Fraction	Pro	S.D.	Asp	S.D.	Glu	S.D.	Phe	S.D.	Lys	S.D.	Tyr	S.D.	Arg	S.D.	Trophic Position
1546	RBC	24.8		20.9		24.3		14.2		12.2		9.4		16.7		2.3
1502	RBC	21.6	2.1	19.7	0.5	24.0	0.9	12.3	0.6	6.4	3.2	11.9	0.7	10.7	3.5	2.5
1458/59	RBC	22.9	2.5	17.0	1.0	23.2	0.7	11.8	2.4	2.2	0.5	10.7	1.5			2.5
1580/81	RBC	22.9	0.9	18.8	0.7	25.0	0.7	15.0	0.6	6.3	2.5	13.2	0.7	6.6	1.1	2.3
1547/51	RBC	27.7	1.2	24.8	0.4	28.1	0.6	18.1	0.3	9.5	1.1	15.3	0.8	14.6	2.5	2.3
1546	Serum	34.0	0.4	22.1	1.0	29.5	0.7	16.1	0.5	11.1	0.5	16.3	1.9	20.8	0.8	2.7
1503	Serum	25.9	0.7	19.8	0.6	25.7	0.6	12.3	0.7	15.0	2.4	14.9	1.9	24.3	0.9	2.7
1517	Serum	27.8	0.2	19.4	1.1	26.0	1.0	15.0	2.5	6.1	5.7	13.2	3.1	8.4	2.2	2.4
1498	Serum	25.3	0.3	19.8	1.0	24.7	0.8	13.7	2.0	6.7	1.7	13.0	1.4	12.9	4.5	2.4
1502	Serum	21.9	0.4	18.6	0.4	20.3	0.9	10.3	1.0	9.3	0.3	16.5	1.6	15.6	2.4	2.3
1489	Serum	29.2	1.3	23.8	0.6	27.5	0.7	16.2	0.9	10.7	1.8	15.8	0.7	13.5	2.2	2.5
1492	Serum	26.0	1.5	20.5	1.7	25.4	1.2	16.7	0.5	17.6	8.4	11.9	1.6			2.1
1490	Serum	29.1	0.5	23.7	0.8	27.5	0.3	15.5	1.3	11.1	0.7	16.8	1.0	11.1	2.3	2.6
1542/43	Serum	28.7	1.0	23.2	1.5	21.7	1.5	13.4	3.0	9.1	1.3	13.2	0.7			2.1
1578/79	Serum	23.6	0.5	18.1	0.4	25.5	0.8	12.8	1.9	6.5	1.2	10.8	1.2			2.7
1458/59	Serum	25.0	2.6	19.8	0.3	24.4	0.7	14.0	1.4	11.4	1.8	16.0	0.1	16.8		2.4
1460/61	Serum	24.7	1.4	16.2	1.3	15.6	1.6	12.2	1.8							1.4
1445/1588	Serum	26.7	1.6	18.9	2.2	23.2	0.6	14.5	0.8	8.5	1.9	14.2	1.0	16.1	1.3	2.1
1456/57	Serum	24.3	0.9	20.5	1.2	24.7	1.3	10.8	0.4	8.8	3.7			-3.0		2.8
1598/99	Serum	27.9	2.9	18.2	1.0	21.4	2.6	13.3	3.6	18.3	2.2	7.3	2.9			2.1
1580/81	Serum	26.7	0.2	19.6	0.4	26.1	0.6	14.4	1.0	10.9	0.8	13.5	1.6	15.4	0.8	2.5
1590/91	Serum	26.5	0.7	17.1	1.1	26.3	1.1	12.3	0.5	10.8	0.4	14.0	0.9	11.5	3.2	2.8
1592/93	Serum	25.5	0.9	16.4	1.1	23.9	0.3	15.3	0.4	4.2	1.4	12.1	2.1	10.7	1.8	2.1
1519/20	Serum	28.2	0.2	17.7	0.9	22.6	0.9	11.9	1.2	7.9	3.9	14.9	5.0			2.4
1574/75	Serum	26.4	4.2	19.7	0.4	23.1	1.0	16.1	0.7	15.4	2.0	14.7	0.3	22.1	0.5	1.9
1594/95	Serum	29.8	0.5	18.0	0.7	23.2	0.7	12.8	1.8	14.9	5.9	14.0	2.5	14.7	4.5	2.4
1596/97	Serum	31.1	1.2	23.0	1.3	25.8	1.3	8.5	1.3	8.2	1.2	19.9	0.0	10.8	1.4	3.3
1547/51	Serum	32.9	0.9	22.4	1.2	27.6	2.0	17.9	1.3	13.3	0.2	17.0	0.8	22.9	0.8	2.3
1576/77	Serum	28.9	0.9	20.9	0.5	24.7	0.5	14.0	0.8	5.9	1.0	11.3	0.2			2.4
1450/51	Serum	28.2	0.6	20.2	1.6	24.3	0.8	9.9	1.0	6.4	2.2	15.5	0.2	14.5	1.3	2.9
1657/58	Serum	27.7	2.0	17.8	0.4	24.1	0.5	14.5	2.1	8.4	2.4	12.4	1.2	14.3	1.1	2.2

Supplementary Table 3.3: δ^{15}N-AA of captive, harbor seals at The Marine Mammal Center. δ^{15}N-AA values of captive harbor seals in both serum and corrected serum fractions. Each AA was injected four times. Sex: Male (M), Female (F); Age: Adult (A), Pup (P), Fetus (F); Status: Released (R), Death (D), Euthanized (E). Health status based off of blubber thickness either right before release of determined after death.

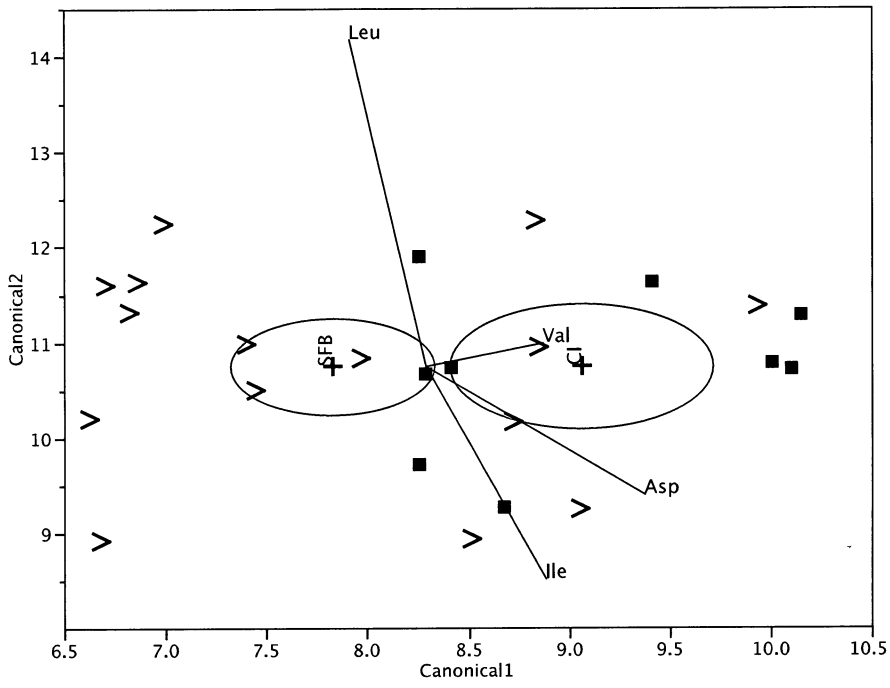
Name	ID#	Fraction	Sex	Age	R.D.E.	Days in Center	Health Status	δ^{15}N (‰) Death
Nigel	1696	Serum	M	P	R	62	Healthy	
Hang Ten	1698	Serum	F	P	R	83	Healthy	
BMB	1704	Serum	F	P	D	36	Healthy	Colitis
Luka	1705	Serum	F	P	R	93	Healthy	
Nabby	1718	Serum	F	P	E	62	Healthy	Neurological
Stumpy May	1739	Serum	F	P	E	18	Healthy	Aspiration; Enteritis
Shenanigans	1748	Serum	M	P	E	1	Healthy	Spine Trauma
Stefan	1707	Corrected Serum	F	A	E	0	Stressed	Septicemica; Pyothroax
Carcass	1726	Corrected Serum	F	F	D	0	Healthy	Trauma - skull
Carcass	1734	Corrected Serum	F	P	D	0	Stressed	Malnutrition; Fat atrophy

Name	ID#	Fraction	Sex	Age	Ala	Gly	Thr	Ser	Val	Leu	Ile	Pro	Asp	Glu	Phe	Lys	Tyr	Arg	Bulk
Nigel	1696	Serum	M	P	27.2	12.9	-17.0	9.1	23.5	24.0	15.5	28.1	18.1	23.7	9.5	14.0	20.3	17.7	16.4
Hang Ten	1698	Serum	F	P	24.1	10.3	-25.4	9.7	17.7	23.7	19.4	29.4	18.7	23.4	10.2	13.5	19.0	18.6	16.5
BMB	1704	Serum	F	P	22.1	12.7	-22.8	10.8	19.7	23.2	22.7	28.7	17.5	22.7	10.7	5.6	19.3		15.8
Luka	1705	Serum	F	P	23.3	9.6	-27.5	9.1	17.1	23.3	21.5	28.3	18.2	23.3	9.6	11.5	17.3	15.8	16.3
Nabby	1718	Serum	F	P	21.9	10.2	-18.6	9.6	22.1	22.8	18.2	26.8	18.8	24.4	9.1	7.0	19.6		15.4
Stumpy May	1739	Serum	F	P	20.1	15.2		13.0	16.7	17.1		21.4	16.2	19.7	11.9	3.5	20.7	9.2	12.4
Shenanigans	1748	Serum	M	P	26.8	18.9	-21.4	19.8	26.6	25.8	20.8	29.0	21.4	25.8	12.7	16.7	14.1		18.0
Stefan	1707	Corrected Serum	F	A	28.8	12.6	-25.7	15.3	28.1	29.5	28.3	31.8	24.1	30.3	18.8	13.8	23.9		19.6
Carcass	1726	Corrected Serum	F	F	25.1	11.1	-20.8	12.3	23.9	26.2	25.8	31.3	22.2	28.3	14.4	12.1	16.9		18.2
Carcass	1734	Corrected Serum	F	P	28.1	13.3	-16.1	13.7	26.6	29.1	25.3	33.4	24.2	29.2	18.1	10.5	22.8		18.4

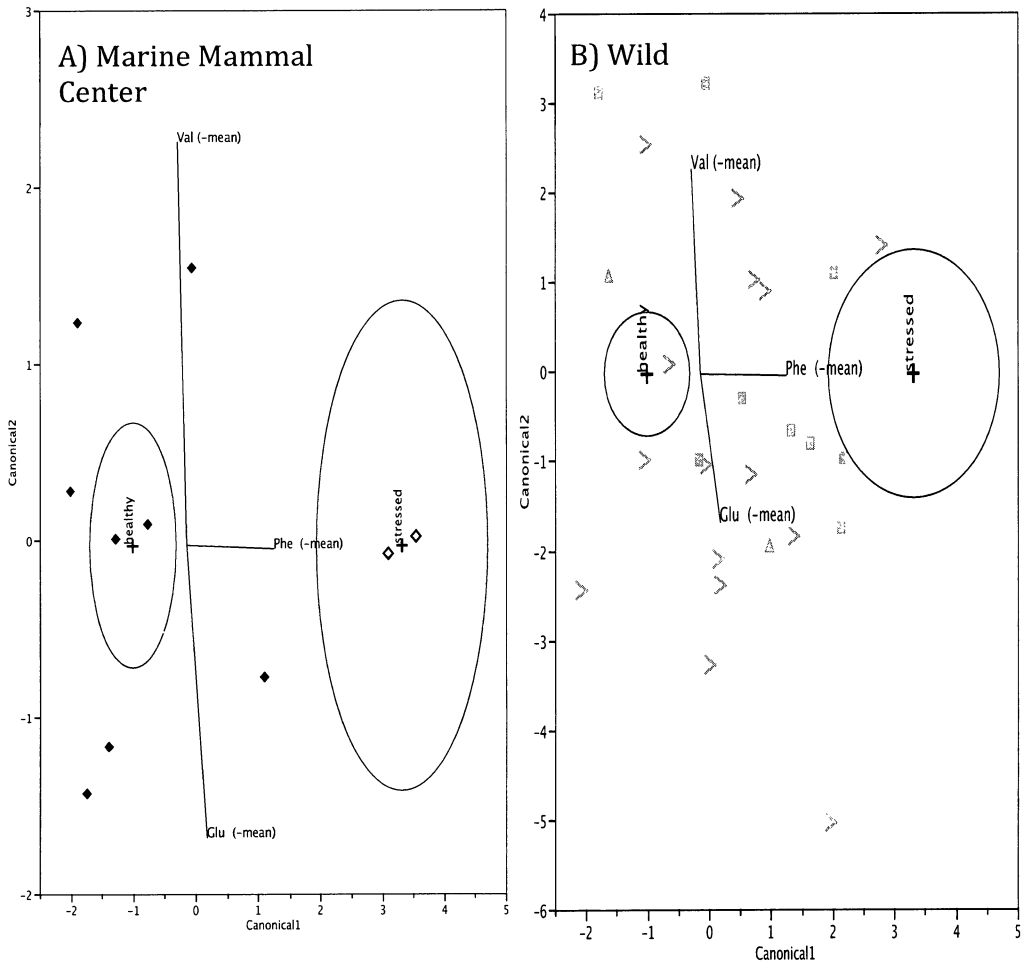
Supplementary Table 3.4: ANOVA for significant differences in individual δ^{15}N-AA in healthy vs. stressed harbor seals. Rehabilitated harbor seals from the Marine Mammal Center (Sausalito, CA) were classified as "healthy" (n=8) vs. "stressed" (n=2) based on veterinary evaluation. δ^{15}N-AA values that differed significantly between the two groups ($P < 0.05$) were chosen for DFA. AA abbreviations are as in text; F-ratio and *P* value refer to ANOVA statistics. DFA predicts that a given AA can be used to separate "stressed" vs. "healthy" seals at 95% confidence.

AA	F-ratio	*P* value
Ala	6.4	0.04
Asp	12.9	0.01
Glu	10.0	0.01
Ile	6.1	0.04
Leu	8.6	0.02
Pro	4.7	0.06
Val	5.6	0.05
Gly	0.0	0.89
Lys	0.2	0.64
Ser	1.1	0.32
Tyr	5.0	0.03
Phe	29.4	0.00
Arg	9.2	0.02
Thr	0.1	0.77

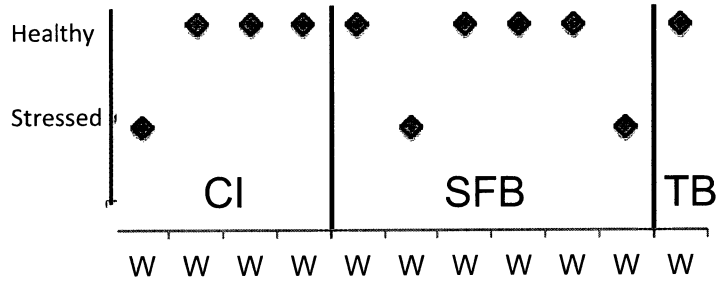
Supplementary Figure 3.1: DFA separation between CI and SFB. Significant AA chosen by ANOVA (Asp, Ile, Leu, Val all had $P < 0.05$) and separated into location groupings (CI = squares; SFB = >).



Supplementary Figure 3.2: DFA to predict nutritional stress status for rehabilitated seals in Marine Mammal Center vs. harbor seals in wild. Refer to Supp. Table 4 for significant AA used in this analysis (Phe, Glu, Val). A) Known nutritional stress DFA separation for seals at the Marine Mammal Center (healthy, solid diamonds, n=8; stressed, open diamonds n=2). B) Predicted nutritional stress status for wild seals (CI = squares; SFB = >; TB = triangles) compared to Marine Mammal Center significant AA values.



Supplementary Figure 3.3: Predicted nutritional stress status for individual, weaner wild harbor seals. Healthy or stressed individual DFA predictions plotted by location (CI = Channel Islands; SFB = San Francisco Bay; TB = Tomales Bay).



Supplementary Methods & Results and Discussion Text 3:

3.1 Preliminary Investigation of Nutritional Stress using DFA

Statistical analyses were preformed in JMP (ver.9) and Matlab. ANOVA was used to assess if there were significant variations in CSI-AA values between the CI and SFB populations. ANOVA was also used to perform a similar calculation using the previously published "healthy" vs. "stressed" data for captive seals (Germain et al, *in review*; *see supplementary material*). For all ANOVAs, a significant result was reported if *P* values were less than 0.05 (Supp. Table 4). There was no significance for δ^{13}C-AA differences. Predictive discriminant function analysis (DFA) was used to assess possibly nutritional stress based on AAs identified as having significant *P* values. A separate post-hoc DFA test, based on result from captive seal data, was also used to test for possible variation in nutritional between CI and SFB locations.

3.2 Statistical analysis of δ^{15}N-AA to predict nutritional stress in individual seals.

Harbor seals in SFB are proposed be directly impacted by human disturbances, particularly by a poor quality and/or quantity of prey items available for consumption. These undesirable dietary conditions could be the result of overfishing in the bay, introduction of invasive species, and pollution. To assess whether individual seals are experiencing nutritional stress, we compare the SFB population to a relatively healthy population living in CI, which lacks most human

disturbances. Only five AAs had $\delta^{15}N$ values significantly different from each other between both locations (ANOVA $P < 0.05$): Asp, Ile, Leu, Val, and Thr, in order of decreasing significance. However, since Thr is typically an AA that behaves uniquely compared to all others (extreme ^{15}N depletion), we excluded it from discriminant function analysis (DFA). DFA is a statistical tool used to amplify separation in groups of data, in this case, SFB seals vs. CI seals, by using canonical scores of significantly reported AAs (Supp. Fig. 1). By using this analysis, if a harbor seal is predicted to be from a different location (*i.e.*, seal from SFB rookery but predicted to be from CI), then that suggests that an individual seal is living under different conditions than the other seals in that same location. By using DFA, out of the nine seals from CI, four of them were placed in the SFB grouping (2 SA and 2 W age classes). Whereas, out of the fifteen SFB seals, five of them were placed in the CI grouping (1 A, 1 SA, and 3 W age classes). In both locations, 50% of weaners were falsely identified. These seals appear to either be consuming a different diet than seals living in nearby rookeries, or they might be experiencing nutritional stress. In the future, these specific AAs need to be tested with animals under direct nutritional stress conditions to determine if they can be potential indicators of health status.

Correspondingly, we attempted a direct nutritional stress study by comparing our $\delta^{15}N$ from wild seals to captive, rehabilitated seals from the Marine Mammal Center (Supp. Table 3). The seals were placed in two categories: healthy

(n=8) or stressed (n=2), established by their condition directly before release back to the wild, or if seal was euthanized, based off blubber thickness and necropsy lab results (Germain et al., *in review*). Since the sample set was quite small and over-represented in the healthy grouping, any DFA predictions will be in favor of a 'healthy' prediction. A combination of a normalized histogram of each AA in the captive seals and significant ANOVA ($P < 0.05$) resulted in the best separation between the AAs: Glu, Val, and Phe (Supp. Fig. 2A; 100% correct prediction for captive seals). By using DFA with these three significant AAs, we predicted the health status of individual seals at all locations (Supp. Fig. 2B; CI, SFB, and TB). In CI, almost all older and yearling seals were predicted to be experiencing nutritional stress (Supp. Fig. 3), while the weaners were healthy, however, this is highly unlikely in this location as they live in less disturbed, oceanic conditions. While in SFB, only yearlings and weaner seals were predicted to be grouped with those experiencing nutritional stress (Supp. Fig. 3; 33% of seals < 2 yr). These younger seals do not travel the distances or depths as the older population, and are more restricted to foraging in the bay, which might have smaller fish and more invertebrates than the outer bay and ocean. Although this fits better with our theory of SFB seals consuming lesser quality prey items, this analysis is still insufficient to accurately predict individual health status of these harbor seals. It is important to note the significant AAs chosen using both types of DFA resulted in different AA selections. Thus, while there do appear to be statistical differences in

some AAs between populations that might be an indicator of some kind of nutritional stress, there still needs to be more directed studies on effects of stress on AA patterns to really interpret the findings.