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Title

Amino acid isotope discrimination factors for a carnivore: physiological insights from leopard sharks and their diet

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[†]Contributions: SLK and PLK formulated the idea, SLK conducted the feeding experiment, all authors contributed to the development of the compound-specific stable isotope analyses, JPW, SLK, and SDN performed laboratory analyses, all authors interpreted the data, and JPW led the collaborative writing of the manuscript.

Abstract

Stable isotopes are important ecological tools because the carbon and nitrogen isotopic composition of consumer tissue reflects the diet. Measurements of isotopes of individual amino acids can disentangle the effects of consumer physiology from spatiotemporal variation in dietary isotopic values. However, this approach requires knowledge of assimilation patterns of dietary amino acids. We reared leopard sharks (*Triakis semifasciata*) on diets of squid (*Loligo opalescens*; 1250 days; control sharks) or squid then tilapia (*Oreochromis* sp.; switched at 565 days; experimental sharks) to evaluate consumer-diet discrimination factors for amino acids in muscle tissue. We found that control sharks exhibited lower nitrogen isotope discrimination factors ($\Delta^{15}\text{N}$) than most previous consumer studies, potentially because of urea recycling. Control sharks also had large carbon isotope discrimination factors ($\Delta^{13}\text{C}$) for three essential amino acids, suggesting microbial contributions or fractionation upon assimilation. Compared to controls, experimental sharks exhibited higher $\Delta^{13}\text{C}$ values for four amino acids and $\Delta^{15}\text{N}$ values for seven amino acids, corresponding with differences between diets in $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values. This suggests that not all amino acids in experimental sharks had reached steady state, contrary to the conclusion of a bulk isotope study of these sharks. Our results imply that 1) the magnitude of a shift in dietary $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values temporarily influences the appearance of discrimination factors; 2) slow turnover of amino acid isotopes in elasmobranch muscle precludes inferences about seasonal dietary changes; and 3) elasmobranch discrimination factors for amino acids may be affected by urea recycling and microbial contributions of amino acids.

Key words:

elasmobranchs, fractionation, growth, microbes, urea

Introduction

Understanding the foraging ecology of upper trophic level consumers, such as elasmobranchs, is important because they can profoundly influence ecosystems (Young et al. 2015; Bird et al. 2018). Researchers often assess elasmobranch diet by analyzing the stable carbon ($\delta^{13}\text{C}$) and nitrogen ($\delta^{15}\text{N}$) isotope values of their bulk tissue (e.g., muscle), which reflect the isotopic composition of the food they assimilate (Hussey et al. 2011; Shiffman et al. 2012). However, temporal and spatial variation in $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values of producers at the base of the food web can confound interpretations about food assimilation (Vokhshoori and McCarthy 2014; Lorrain et al. 2015). Analysis of the isotopic composition of individual amino acids is a potentially useful technique to resolve these confounding factors because amino acid metabolism provides a context for data interpretation (e.g., for many consumers, certain amino acids can only be obtained from the diet while others can be synthesized *de novo*). However, this approach requires understanding amino acid discrimination factors (Δ), which are offsets in amino acid $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values between consumers and their diet (e.g., $\Delta^{15}\text{N} = \delta^{15}\text{N}_{\text{consumer}} - \delta^{15}\text{N}_{\text{diet}}$). These discrimination factors in sharks may differ from those reported for other organisms because of their unusual physiology as carnivorous ectotherms who retain urea for use as an osmolyte (Hussey et al. 2010; Hoen et al. 2014; Kim et al. 2012b).

Initial biological applications of stable isotope analysis often used the simplifying assumption that dietary nutrients were assimilated as homogenous pools of carbon and nitrogen, from which macromolecules could be synthesized (Martínez del Río et al. 2009). However, some dietary macromolecules are assimilated intact. For instance, animals cannot synthesize essential amino acids (e.g., threonine, phenylalanine, lysine, isoleucine, leucine, valine) and thus their

carbon skeletons are routed directly into endogenous tissue from dietary protein, leading to relatively small carbon discrimination factors (Howland et al. 2003; Jim et al. 2006). As a result, the $\delta^{13}\text{C}$ values of essential amino acids in consumer tissues reflect those of primary producers in a food web. In contrast, non-essential amino acids (e.g., glycine, serine, alanine, aspartic acid, glutamic acid, tyrosine) can be synthesized *de novo* by animals, potentially from non-protein carbon sources (e.g., lipids and carbohydrates). Carbon isotopic discrimination factors for these amino acids, therefore, may be larger and more variable, reflecting complex biochemical pathways (McMahon et al. 2010; Newsome et al. 2014).

Nitrogen isotope dynamics for amino acids do not necessarily align with the categories of essential and non-essential. Instead, some amino acids retain their amine nitrogen through metabolic processing. These “source amino acids” (e.g., phenylalanine, lysine, tyrosine; and in some organisms, glycine and serine) exhibit relatively small isotope discrimination factors, meaning they preserve the $\delta^{15}\text{N}$ values of primary producers across a food web (Chikaraishi et al. 2007; McMahon and McCarthy 2016). In contrast, “trophic amino acids” (e.g., alanine, aspartic acid, glutamic acid, isoleucine, leucine, valine) routinely exchange amine nitrogen with a consumer’s internal nitrogen pool after absorption, leading to large discrimination factors that cause $\delta^{15}\text{N}$ to increase with trophic level (Nielsen et al. 2015; O’Connell 2017). The magnitude of discrimination factors is also influenced by nitrogen use efficiency (Cantalapiedra-Hijar et al. 2017) and nutritional composition of the diet. Diets with amino acid compositions more similar to the tissue of the consumer often result in smaller discrimination factors, for both bulk tissue and individual amino acids (Robbins et al. 2005; Florin et al. 2011; McMahon et al. 2015). Total dietary protein also affects bulk discrimination factors, although contradictory trends have been reported with high protein correlating with both increased (Kelly and Martínez del Río 2010) and

decreased discrimination (Hughes et al. 2018). Thus, discrimination factors for bulk tissue and amino acids of consumers can be influenced by their diet, physiology, and trophic level.

Discrimination factors are often assessed with controlled feeding experiments using captive animals. Such experiments must also consider isotopic turnover rate, which is the pace of incorporation of dietary carbon and nitrogen into consumer biomass during tissue addition (i.e., accretion during growth) or replacement (i.e., maintenance). Turnover rate is influenced by metabolic rate and body size, among other factors (Martínez del Río and Carleton 2012; Vander Zanden et al. 2015). Most elasmobranchs are ectotherms, which tend to exhibit slow turnover rates (Vander Zanden et al. 2015), and sharks can take over a year for their bulk muscle tissue to reach a steady state with the isotopic composition of a new diet (Malpica-Cruz et al. 2012; Kim et al. 2012b). Importantly, incomplete turnover can cause discrimination factors to appear different than they would for an animal that has reached steady state (Fig. 1).

Here, we use a long-term feeding study to evaluate variations in carbon and nitrogen isotope discrimination factors for amino acids in muscle tissue from leopard sharks (*Triakis semifasciata*), an abundant predator in Pacific coastal waters of North America. Nine captive leopard sharks were fed squid (*Loligo opalescens*) for 565 days then divided into control and experimental groups (Kim et al. 2012a, b). For the subsequent 685 days, control sharks (N = 3) continued eating squid while experimental sharks (N = 6) were fed tilapia (*Oreochromis* sp.). Bulk tissue analysis of muscle samples collected at the end of the experiment indicated that $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ of both control and experimental sharks had reached steady states with their respective diets (Kim et al. 2012a, b), although bulk $\Delta^{13}\text{C}$ and $\Delta^{15}\text{N}$ differed between diets. Using the same set of muscle samples, here we test the fundamental assumptions that isotopic discrimination factors are 1) near zero for essential amino acids ($\Delta^{13}\text{C}$) and source amino acids ($\Delta^{15}\text{N}$), and 2)

larger than zero for non-essential amino acids ($\Delta^{13}\text{C}$) and trophic amino acids ($\Delta^{15}\text{N}$). We also test whether diet amino acid concentrations influenced discrimination factors. Finally, although we expected that all amino acids in muscle from both groups of sharks had reached isotopic steady states with their diets, we evaluated whether variation in discrimination factors was suggestive of incomplete turnover. The results provide a framework for interpreting the movement and foraging ecology of wild elasmobranchs using amino acid isotope data.

Materials and Methods

Feeding experiment and sampling. Details of husbandry, feeding, and sampling for this controlled feeding study can be found in Kim et al. (2012a, b) and Zeichner et al. (2017). Briefly, nine juvenile leopard sharks were caught in San Francisco Bay between August–December 2005 via otter trawl with the Marine Science Institute (Redwood City, CA) and housed at the Long Marine Lab of the University of California, Santa Cruz throughout the experiment. Sharks were kept in polyethylene tanks (1–2 individuals per tank; 2.3 m diameter and 1.2 m water depth) with a continuous flow of filtered seawater from Monterey Bay. All sharks were fed three times per week throughout the experiment; individuals sharing tanks were separated by a net for feeding. All sharks (both the squid and tilapia fed) received 3–5% of their body mass per day, which was adjusted throughout the experiment. Every 2–3 weeks, total body length was measured and serial sampling of tissue was performed for other studies (i.e., blood, muscle, and teeth). Hematocrit values were periodically assessed as a measure of health. Starting on day 0 of the experiment (13-January-2006) all individuals were fed a constant diet of squid. On day 565 (01-August-2007), six individuals (in three tanks) were randomly switched to the experimental group and thereafter fed tilapia (farm-raised in Taiwan) for a subsequent 685 days. The remaining three

individuals in the control group continued to receive squid for the entire 1250 days. Squid and tilapia were ordered in a single batch at the beginning of the experiment and portioned once per month as whole squid or headless tilapia. On day 1250 (01-July-2009), all sharks were sacrificed using MS-222 and muscle samples were collected. The present study only includes these shark muscle samples collected at the end of the experiment. Samples of shark muscle, whole squid, and headless tilapia were freeze-dried and stored in plastic bags until isotopic analysis. The care, sampling, and sacrifice protocol for the sharks in this study was approved by the UC Santa Cruz Chancellor's Animal Research Committee (CARC), in accordance with Institutional Animal Care and Use Committee (IACUC) standards (permit # Kochp0901).

Sample preparation and elemental and isotopic analyses. Bulk tissue measurements of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values for all shark muscle samples (Table S1) and diet items were analyzed in Kim et al. (2012a, b). Samples of squid and tilapia were analyzed for amino acid composition at the University of Wyoming (WY, USA) Macromolecular Analysis Core on an AB Sciex TOF/TOF 5800 mass spectrometer. Samples of muscle from control sharks were analyzed for amino acid composition at the University of California, Santa Cruz (CA, USA) on a quadrupole gas chromatograph-mass spectrometer (Agilent 7890A GC coupled to MS 5975B/EI); results were reported in Kim and Koch (2012). We assumed that the amino acid composition of these muscle samples from control sharks was representative of experimental sharks as well because muscle amino acid composition is well-conserved among shark species (Chandrashekar and Deosthale 1993; Onodenalore and Shahidi 1996; Diniz and Martin 1997) and because individuals in both groups ate consistently, grew in length, and had adequate hematocrit values throughout the experiment, suggesting that there were no major physiological stressors.

All other analyses were performed at the Center for Stable Isotopes at the University of New Mexico (NM, USA). Two subsamples of squid and two subsamples of tilapia were weighed into tin capsules (~0.5 mg) and analyzed for percent carbon and nitrogen on a Costech 4010 Elemental Analyzer. Other subsamples of squid and tilapia, as well as all shark muscle samples, were lipid-extracted by soaking them three times with 2:1 chloroform:methanol (24 hours per soak) then rinsing them four times with distilled water. Shark muscle samples were then additionally soaked three times with distilled water (24 hours per soak) to remove urea (Kim and Koch 2012). All samples were then freeze-dried for 24 hours.

Isotope analysis of individual amino acids followed Engel and Hare (1985) and Fantle et al. (1999). Samples were weighed out to approximately 10–15 mg and hydrolyzed in 1 ml of 6N HCl for 20 hours at 110°C. During hydrolysis, glutamine was converted to glutamic acid and asparagine was converted to aspartic acid. Hydrolyzed samples were dried under a stream of N₂ gas then derivatized to N-trifluoroacetic acid isopropyl esters and resuspended in dichloromethane. Samples were injected (1 µl) into a gas chromatograph (Thermo Scientific Trace 1300 GC; column BPx5, 60 m) for separation of amino acids, which were then combusted to CO₂ or reduced to N₂ at 1000°C (Thermo Scientific GC Isolink II) and analyzed on an isotope ratio mass spectrometer (Thermo Scientific Delta V Plus IRMS).

For each sample, we analyzed two injections for $\delta^{13}\text{C}$ values (with a standard every fifth injection) and three injections for $\delta^{15}\text{N}$ values (with a standard every fourth injection). SD for multiple injections of the same sample averaged 0.16‰ (range 0.00–0.62) for $\delta^{13}\text{C}$ and 0.55‰ (0.05–1.92) for $\delta^{15}\text{N}$ values. Standards of pure amino acids of known isotopic composition (Sigma-Aldrich Co.) had SDs for multiple injections that averaged 0.28‰ (0.00–1.21) for $\delta^{13}\text{C}$

and 0.81‰ (0.00–1.69) for $\delta^{15}\text{N}$ values. Standardization of runs was achieved using intermittent pulses of CO_2 or N_2 gases of known isotopic value.

To account for the addition of carbon and the kinetic isotope fractionation associated with derivatization, $\delta^{13}\text{C}$ values were corrected as follows:

$$\delta^{13}\text{C}_{\text{sample.underiv}} = (\delta^{13}\text{C}_{\text{sample.deriv}} - \delta^{13}\text{C}_{\text{standard.deriv}} + (\delta^{13}\text{C}_{\text{standard.underiv}} \times P)) \times P^{-1} \quad (\text{eq. 1})$$

Here, $\delta^{13}\text{C}_{\text{sample.underiv}}$ is the final, calculated value of the amino acid; $\delta^{13}\text{C}_{\text{sample.deriv}}$ is the measured value of the derivatized amino acid; $\delta^{13}\text{C}_{\text{standard.underiv}}$ is the measured value of the un-derivatized amino acid in the standard (previously assessed via elemental analyzer coupled with isotope ratio mass spectrometry); and P is the proportion of carbon in the amino acid from the original sample. Correction of $\delta^{15}\text{N}$ values was less complex because derivatization does not add exogenous nitrogen:

$$\delta^{15}\text{N}_{\text{sample.underiv}} = (\delta^{15}\text{N}_{\text{sample.deriv}} + (\delta^{15}\text{N}_{\text{standard.deriv}} - \delta^{15}\text{N}_{\text{standard.underiv}})) \quad (\text{eq. 2})$$

This method of analysis yields data on 13 amino acids. However, here we exclude stable isotope data from proline because its values are affected by co-elution with hydroxyproline.

Statistical analyses. For all sharks, the discrimination factor for each amino acid ($\Delta^{15}\text{N}_{\text{shark muscle - diet}}$ and $\Delta^{13}\text{C}_{\text{shark muscle - diet}}$) was compared to zero using a one-tailed t-test. In addition, amino acid discrimination factors were compared between control and experimental sharks using a two-tailed t-test or if the data failed to exhibit normality (via the Shapiro-Wilk test), a Mann-Whitney rank test. To evaluate the influence of amino acid composition on discrimination factors, linear regressions were analyzed in which the predictor variable was amino acid imbalance (i.e., the mole percent of an amino acid in the diet minus the mole percent of that amino acid in shark muscle) and the response variable was the mean discrimination factor for that amino acid (carbon or nitrogen) between sharks and their diet. In general, $\Delta^{13}\text{C}$ values

should be larger for non-essential than for essential amino acids and $\Delta^{15}\text{N}$ values should be larger for trophic than for source amino acids. Thus, regression models were evaluated using all data; using only non-essential amino acids for $\Delta^{13}\text{C}$ values; and using only trophic amino acids for $\Delta^{15}\text{N}$ values. Linear regressions were used to assess the relationship between 1) the difference between diets in $\delta^{13}\text{C}$ or $\delta^{15}\text{N}$ values, and 2) the difference in $\Delta^{13}\text{C}$ or $\Delta^{15}\text{N}$ values between sharks consuming each diet. Residual normality of regression models was assessed with the Shapiro-Wilk test and potential outliers were evaluated with Cook's Distance score. The α value was 0.05 for all tests. Statistical analyses were calculated in SigmaPlot 13.0.

Results

Comparisons of nutritional composition of diets. The control diet of squid and the experimental diet of tilapia were relatively similar in nutrition. Mean C:N ratios of non-lipid-extracted samples suggested that squid (3.7 ± 0.2 SD; $n = 2$) was lower in fat than tilapia (4.5 ± 0.2 ; $n = 2$). Using an empirically-derived equation to convert C:N ratios to percent lipid by mass for aquatic organisms (equation 2 in Post et al. 2007), the squid diet was 6% fat ($\pm 1\%$ SD) and the tilapia diet was 12% ($\pm 1\%$ SD). The amino acid composition (mole percent) of squid, tilapia, and shark muscle appeared to be similar (Table 1). Amino acid imbalances between shark muscle and diet items did not correlate to amino acid discrimination factors for carbon or nitrogen (Table 2), with one exception: the imbalance among non-essential amino acids correlated to $\Delta^{13}\text{C}$ values for experimental sharks. However, a high Cook's Distance score (>1) suggested that serine was an outlier in this model and after its removal, this correlation was not significant (Table 2). Bulk isotope values of lipid-extracted diet samples, analyzed and presented in Kim et al. (2012b),

differed for $\delta^{13}\text{C}$ values (squid: $-18.5 \pm 0.5\text{‰}$ SD, tilapia: $-23.2 \pm 0.9\text{‰}$) and $\delta^{15}\text{N}$ values (squid: $13.3 \pm 0.7\text{‰}$, tilapia: $7.9 \pm 0.4\text{‰}$).

Carbon isotope discrimination. Amino acids varied in $\delta^{13}\text{C}$ values for squid, tilapia, and shark muscle (Fig. 2a). Both experimental and control sharks exhibited a wide range of amino acid discrimination factors (Fig. 2b), although several $\Delta^{13}\text{C}$ patterns were similar between these two groups. Among non-essential amino acids, both experimental and control sharks exhibited a discrimination factor for serine that did not differ from zero. In contrast, both groups exhibited large discrimination factors for the ketogenic amino acids (alanine, aspartic acid, glutamic acid, tyrosine). Among essential amino acids, both experimental and control sharks showed no discrimination for threonine but high discrimination for leucine and valine. Discrimination factors for other amino acids differed between groups. One non-essential (glycine) and three essential (phenylalanine, lysine, and isoleucine) amino acids had larger discrimination factors for experimental sharks than for control individuals (Fig. 2b). The differences in $\Delta^{13}\text{C}$ values between experimental and control sharks correlated with the $\delta^{13}\text{C}$ differences between their respective diet items (tilapia and squid; Fig. 3a). Assuming that the control sharks were in a steady state with their diet (see Discussion) and based on the similar nutrition of the two diets, it is expected that the experimental sharks would eventually exhibit the same amino acid $\Delta^{13}\text{C}$ values as control sharks. Thus, amino acids that did not differ in $\Delta^{13}\text{C}$ between control and experimental sharks either had similar $\delta^{13}\text{C}$ values between diets or had turned over $\sim 100\%$ of their carbon pool in the experimental sharks. In contrast, the amino acids that differed in $\Delta^{13}\text{C}$ between control and experimental sharks were calculated to have turned over 36–64% of their carbon pool (Table 3).

Nitrogen isotope discrimination. Amino acids varied in $\delta^{15}\text{N}$ values for squid, tilapia, and shark muscle (Fig. 4a). For most source amino acids, $\delta^{15}\text{N}$ values were similar for squid, tilapia, experimental shark muscle, and control shark muscle, resulting in relatively small amino acid discrimination factors between diet and consumer (Fig. 4b). For both experimental and control sharks, discrimination did not differ from zero for phenylalanine and median values were $<3\text{‰}$ for lysine and tyrosine. Between groups, median $\Delta^{15}\text{N}$ values were similar for glycine (1–2‰) and dissimilar for serine (2‰ for experimental, -5‰ for control). Discrimination factors for threonine and for trophic amino acids were greater for the experimental group than for the control group. For experimental sharks, trophic amino acids had a mean discrimination factor of 9.8‰ (± 1.6 SD), while the mean for control individuals was 3.8‰ (± 1.4). The discrimination factor for threonine was less for experimental sharks (median of -11‰) than for control sharks (median of -8‰). The differences in $\Delta^{15}\text{N}$ values between experimental and control individuals correlated with $\delta^{15}\text{N}$ differences between their respective diet items (tilapia and squid; Fig. 3b). The Cook's Distance score indicated that threonine was a potential outlier in this relationship; however, regression statistics were almost unchanged after its removal ($P < 0.01$, $R^2 = 0.95$). Similar to carbon, it is expected that the experimental sharks would eventually exhibit the same amino acid $\Delta^{15}\text{N}$ values as control sharks. Thus, amino acids that did not differ in $\Delta^{15}\text{N}$ between control and experimental sharks either had similar $\delta^{15}\text{N}$ values between diets or had turned over ~100% of their nitrogen pool in the experimental sharks. In contrast, the amino acids that differed in $\Delta^{15}\text{N}$ between control and experimental sharks were calculated to have turned over 17–47% of their nitrogen pool (Table 3).

Discussion

The stable isotope analysis of individual amino acids has created new opportunities to study the foraging ecology of upper trophic level consumers, such as elasmobranchs. However, application of this technique requires careful calibration of isotopic discrimination factors of amino acids, via controlled feeding experiments if possible. For many amino acids in our study, $\Delta^{13}\text{C}$ and $\Delta^{15}\text{N}$ values were larger for experimental sharks (fed squid for 565 days then switched to tilapia for 685 days) than for control sharks (fed squid for the entire 1250 days). This result was surprising because we expected that by the experiment's end, experimental individuals would be in a steady state with their new diet (Kim et al. 2012a, b) and therefore they would exhibit similar amino acid discrimination factors as control individuals for both carbon and nitrogen.

Although discrimination factors can be influenced by dietary nutritional composition, this factor was unlikely to have caused differences between experimental and control sharks, for two reasons. First, protein quality appeared to be relatively high for both diets; poor quality is indicated by dissimilarity in amino acid composition between consumer tissue and food correlating with large discrimination factors (McMahon et al. 2015). Here, we found no such correlations for sharks consuming either diet. Second, protein content was likely high for both diet items, consistent with their relatively low C:N ratios. Nutritional assessments show that protein, carbohydrate, and lipid content for squid average 78%, 15%, and 7% and for tilapia average 92%, 0%, and 8% (USDA). The difference in carbohydrates did not appear to affect amino acid metabolism; if it had, we would have expected control sharks to exhibit larger $\Delta^{13}\text{C}$ values because of the greater availability of a non-protein carbon source for amino acid synthesis (Newsome et al. 2011), which we did not observe. Instead, we suggest that the primary cause of differences in amino acid discrimination factors between experimental and control sharks was

incomplete turnover for some amino acids in the experimental treatment, as discussed in detail below.

Experimental sharks. Compared to control sharks, experimental individuals had greater $\Delta^{13}\text{C}$ values for one non-essential (glycine) and three essential amino acids (Fig. 2; phenylalanine, lysine, isoleucine) and greater $\Delta^{15}\text{N}$ values for all six trophic amino acids (alanine, asx, glx, isoleucine, leucine, valine) and threonine (Fig. 4). These differences in discrimination factors could be caused by amino acids in the muscle of experimental sharks having not yet reached a steady state with their new diet. This explanation is supported by the facts that 1) the new diet was lower in $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$, which would lead to the observed direction of change in amino acid discrimination factors, and 2) the magnitude of difference in $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values between diets predicted the amount by which the amino acid discrimination factor increased after the diet switch. Larger offsets between diet and consumer $\delta^{13}\text{C}$ or $\delta^{15}\text{N}$ values make it easier to discern a lack of steady state conditions, whereas smaller offsets (especially those that are similar in magnitude to analytical uncertainty) can create a perception of steady state when it has not yet been achieved (Fig. 1). For example, aspartic acid was similar in $\delta^{13}\text{C}$ values between diets: -23‰ for squid and -26‰ for tilapia. Thus, after the switch from squid to tilapia, the aspartic acid of the experimental shark muscle only needed to decline by 3‰ to reach a new steady state, a relatively small difference considering our analytical precision (0.2–0.8‰). By the end of the experiment, aspartic acid appeared to have reached a steady state because its discrimination factor in experimental sharks was nearly identical to that in control sharks. In contrast, the diets had very different glycine $\delta^{13}\text{C}$ values (-6‰ for squid and -16‰ for tilapia), and thus this amino acid had to decline by 10‰ in shark muscle to reach a new steady state. As a result, by the end

of the experiment, it was apparent that glycine had not yet reached the new steady state because its discrimination factor for experimental individuals was still much larger than that of control individuals (by ~5%).

The variable magnitude of the required shift in isotopic values after the diet switch can also contribute to dissociation between carbon and nitrogen dynamics. For example, the carbon in the glycine of muscle in the experimental sharks appeared to have not yet reached a steady state because of the large change required in $\delta^{13}\text{C}$ after the diet switch (10‰). However, the required shift in glycine $\delta^{15}\text{N}$ was much smaller (1‰). Thus, as would be expected, the glycine $\Delta^{15}\text{N}$ value for experimental individuals appeared to be very similar to that of control individuals, giving the appearance that the nitrogen in glycine was close to a steady state with the new (tilapia) diet.

The apparent lack of a steady state for multiple amino acids must be reconciled with the conclusion of Kim et al. (2012b) that the bulk muscle tissue of these same experimental sharks had reached a steady state with their new diet for both carbon and nitrogen. We offer two possible explanations for this discrepancy. First, the ostensibly steady isotope values of the bulk tissue at the end of the experiment may have represented a temporary plateau in isotopic turnover rather than a steady state. Incorporation of dietary isotopes usually does not occur at a uniform rate, but instead depends upon protein turnover and tissue accretion (Carleton and Martínez del Rio 2010). Body length measurements indicate that experimental sharks underwent annual periods of accelerated growth during July–November, coinciding with seasonal increases in the temperature of Monterey Bay seawater, which circulated in the shark tanks (Fig. S1 includes serial measurements of body length and bulk muscle tissue $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ for each shark, and seawater temperature during the experiment). Warmer water temperatures likely increased both

protein turnover and tissue accretion in the sharks (Pauly 1980; Fauconneau and Arnal 1985), providing a mechanism for simultaneous rapid changes in tissue $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values. Kim et al. (2012b) collected their last serial sample during April, several months into a period of cooler water temperatures and relative stasis for both growth and changes in muscle tissue $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values. It is possible that had the study continued serial sampling through the following July–November, warmer water temperatures would have caused further change in bulk muscle $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values of the experimental sharks, removing the appearance of a final asymptote. In such a scenario, bulk isotope data would have indicated that sharks had not yet reached a steady state, consistent with the results that we report here for individual amino acids.

A second explanation is that the amino acid composition of shark muscle led to a bulk tissue isotopic value in experimental individuals, which obscured the lack of a steady state. Previous studies of other shark species (dogfish, *Squalus acanthias*; mako, *Isurus oxyrinchus*; sharphead, *Scoliodon sorrakowah*) indicate that their muscle contains amino acids that we did not measure (tryptophan, proline, hydroxyproline, methionine, cysteine, arginine, histidine, taurine; Chandrashekar and Deosthale 1993; Onodenalore and Shahidi 1996; Diniz and Martin 1997). We assumed that muscle of leopard sharks has similar amino acid composition as these other species. After accounting for the number of carbon and nitrogen atoms in the amino acids which we did not measure, they represent 20–22% of the total carbon and 28–31% of the total nitrogen in the protein of bulk muscle tissue. Thus, the amino acids we measured represent ~80% of the total carbon and ~70% of the total nitrogen in muscle. The amino acids that did not differ in $\Delta^{13}\text{C}$ values between control and experimental sharks had likely reached a steady state in both groups. After accounting for their carbon and nitrogen atoms, these amino acids represent 52% of the total carbon in muscle, while the amino acids that were potentially not in steady state

represent 28%. Thus, at least half of the carbon in the bulk muscle tissue would have given the appearance of a steady state. However, this is a less probable explanation for nitrogen. The amino acids likely in steady state in the experimental sharks provide only 24% of the total nitrogen in muscle, while those not in a steady state provide 46%. Thus, nitrogen in the bulk muscle tissue should have been more likely to represent incomplete isotopic turnover.

Overall, the magnitude of difference in $\delta^{13}\text{C}$ or $\delta^{15}\text{N}$ values of amino acids between the control (squid) and experimental (tilapia) diets appeared to be the most important influence on differences in amino acid discrimination factors between groups in our study. This dynamic can affect the ability to distinguish between steady state and incomplete turnover, especially for bulk tissue analyses, because they represent the weighted average of isotopic values of all compounds present in a tissue. This finding has important implications for the use of isotopic analysis in captive feeding studies and for inferring diet of free-ranging individuals. To date, only two studies have estimated isotopic incorporation rates of amino acids in marine organisms after a diet switch: Bradley et al. (2014) reported on Pacific bluefin tuna (*Thunnus orientalis*) and Downs et al. (2014) on Pacific white shrimp (*Litopenaeus vannamei*). The time required to replace 95% of endogenous nitrogen varied among amino acids from 214–1836 days in tuna and from 29–411 days in shrimp. Some of this variation may have been caused by $\delta^{15}\text{N}$ differences among amino acids in the original diets prior to the start of the experiments. An amino acid with a $\delta^{15}\text{N}$ value that was similar between old and new diets could appear to have a quicker isotopic incorporation rate than an amino acid that differed substantially in $\delta^{15}\text{N}$ value between diets, even if the turnover rates were identical (e.g., Fig. 1). Lastly, our conclusion that some amino acids in experimental sharks had not reached steady state 685 days after a diet switch implies that this tissue integrates diet information across multiple years. As a result, researchers should

consider that isotopic composition of muscle in sharks and other large, ectothermic marine consumers likely cannot reveal seasonal shifts in diet or habitat use.

Control sharks. We believe that discrimination factors for individual amino acids in control sharks were accurate and not influenced by incomplete incorporation of the diet, for several reasons. First, by the time of sampling at the end of the experiment, control individuals had been on a constant diet for 1250 days and had not exhibited substantial, directional change in bulk tissue $\delta^{13}\text{C}$ or $\delta^{15}\text{N}$ values for >400 days (Kim et al. 2012a). Second, a recent review found that the longest time interval reported for elasmobranch muscle to replace 95% of endogenous carbon or nitrogen was 422 days (Galván et al. 2016). Although we propose that the appearance of steady states may not always be reliable (as described in the previous section), the fact that control sharks consumed the same diet for a period three times longer than the maximum reported interval for 95% turnover makes it likely that they had reached a steady state.

Data from control sharks supported our prediction that discrimination factors would be larger than zero for non-essential amino acids. The largest $\Delta^{13}\text{C}$ values in control sharks were for aspartic acid and glutamic acid, suggesting extensive *de novo* synthesis. This result is consistent with the roles of aspartic acid and glutamic acid as important metabolic intermediates in the processing of nitrogen derived from amino acid catabolism, which is prevalent in hypercarnivores such as the sharks in this study. In addition, aspartic acid and glutamic acid are ketogenic and thus their synthetic pathways most immediately use other amino acids as a carbon source, which would be plentiful for animals consuming a protein-rich diet. In contrast, control sharks exhibited smaller $\Delta^{13}\text{C}$ values for the glycolytic amino acids (glycine, serine, alanine), which are primarily synthesized using carbohydrates as a carbon source, which were relatively

limited in both diets. Glycine and alanine had positive $\Delta^{13}\text{C}$ values, suggesting some *de novo* synthesis, while serine was the only non-essential amino acid that did not support our predictions. Serine had a $\Delta^{13}\text{C}$ value that did not differ from zero, suggesting that a substantial portion of this amino acid in control sharks was routed directly from the diet into muscle.

Among the essential amino acids in muscle of control sharks, threonine, phenylalanine, and lysine supported our prediction that their discrimination factors would not differ from zero. This result indicates that sharks tended to directly route these amino acids into their muscle. Surprisingly, the discrimination factors for isoleucine, leucine, and valine differed from zero. At least two non-exclusive mechanisms could cause this pattern. First, dietary isoleucine, leucine, and valine tend to be oxidized for energy at a higher rate than dietary threonine, phenylalanine, and lysine (Wu 1998). If the degradative enzymes (i.e., the branched-chain alpha-keto acid dehydrogenase complex) preferably catabolize dietary isoleucine, leucine, and valine with ^{12}C atoms, the $\delta^{13}\text{C}$ value of the remaining, assimilated amino acids would increase and the $\Delta^{13}\text{C}$ values would be positive. Second, isoleucine, leucine, and valine are all synthesized by the same biochemical pathway from pyruvate, which is absent in animals. Because sharks in this study were exclusively fed a known diet, positive $\Delta^{13}\text{C}$ values could reflect contribution from symbiotic microbes (Givens et al. 2015). For instance, gut microbes have been shown to play an important role in digestion for bonnethead sharks (*Sphyrna tiburo*; Jhaveri et al. 2015). Although it is counterintuitive that an animal consuming a high-protein diet would rely on microbes for essential amino acids that are incorporated into tissue, this could be related to a role for such microbes in the recycling of urea, as discussed below. Future research should investigate the potential flux of amino acids from microbes to shark hosts, since this process could confound identification of primary producers in a food web based on $\delta^{13}\text{C}$ values of consumer tissue.

In comparison to $\Delta^{13}\text{C}$ values, control sharks exhibited less variation in $\Delta^{15}\text{N}$ values (note that our $\Delta^{15}\text{N}$ results are similar to those of Hoen et al. (2014), who analyzed the same control sharks in a larger study of carnivorous fish; Fig. S2). Trophic amino acids exhibited a mean $\Delta^{15}\text{N}$ value of 3.8‰ (± 1.4 SD), supporting our prediction that these discrimination factors would be larger than zero. Notably, this value is lower than the average $\Delta^{15}\text{N}$ of 5.4‰ from a recent meta-analysis of published trophic amino acid discrimination factors in studies of consumers with controlled or well-constrained dietary sources (McMahon and McCarthy 2016). However, our $\Delta^{15}\text{N}$ values were similar to those reported from controlled feeding experiments with other sharks and a carnivorous fish (opaka-paka; *Pristipomoides filamentosus*; Hoen et al. 2014). Lower than expected $\Delta^{15}\text{N}$ values of trophic amino acids have also been predicted for free-ranging brown stingrays (*Dasyatis lata*) and scalloped hammerhead sharks (*Sphyrna lewini*) to reconcile unrealistically low trophic positions based on a compound-specific approach with higher trophic positions based on stomach content and bulk tissue isotope analysis (Dale et al. 2011). Such low values of $\Delta^{15}\text{N}$ could be caused by a high protein diet, which has been associated with reduced isotopic discrimination in both bulk tissue (Hughes et al. 2018) and individual amino acids (McMahon et al. 2015), potentially because of a reduced need for *de novo* protein synthesis. However, for some amino acids in our study, this explanation conflicts with the simultaneous inference that elevated $\Delta^{13}\text{C}$ values are indicative of extensive *de novo* synthesis. For example, among non-essentials, aspartic acid and glutamic acid exhibited $\Delta^{13}\text{C}$ values of 9–16‰ but $\Delta^{15}\text{N}$ values of only 2–4‰.

We suggest that the relatively small $\Delta^{15}\text{N}$ values of trophic amino acids in the control sharks were not necessarily caused by reduced rates of amino acid synthesis, but instead by recycling of urea nitrogen (Germain et al. 2013; McMahon and McCarthy 2016). In ureotelic

animals, catabolism of amino acids creates a pool of nitrogen, of which ^{14}N is selectively incorporated into urea then excreted. The remaining nitrogen pool becomes relatively enriched in ^{15}N and is used for synthesis of some endogenous amino acids, leading to large $\Delta^{15}\text{N}$ values (Lee et al. 2012). However, sharks retain urea for use as a tissue osmolyte (Ballantyne 1997). This process is so important that some sharks synthesize additional urea by converting ammonia from surrounding seawater (Wood and Giacomini 2016). Sharks, like most vertebrates, likely lack the enzymes for hydrolyzing urea and recycling its nitrogen but can host populations of bacteria capable of urea hydrolysis (Stevens and Hume 1998). Indeed, such populations occur in shark muscle (Grimes et al. 1985) and bacterially-mediated urea breakdown has been demonstrated in shark liver tissue (Knight et al. 1988). Sharks have high rates of amino acid catabolism and urea production, which would typically lead to elevated $\Delta^{15}\text{N}$ values if that urea was excreted; but they retain it and it is highly feasible that sharks then rely on bacterial symbionts to break down the urea and make the nitrogen therein available for re-use. The incorporation of ^{14}N recycled from urea into newly-synthesized amino acids and endogenous tissue could explain the low $\Delta^{15}\text{N}$ values of trophic amino acids in sharks. This assimilation of microbially-produced amino acids could also lead to the non-zero $\Delta^{13}\text{C}$ that we observed for some essential amino acids. Researchers interpreting isotope data from free-ranging elasmobranchs should consider that individuals may recycle urea and exhibit a $\Delta^{15}\text{N}$ lower than expected for an upper trophic level consumer (McMahon and McCarthy 2016). Unless this potential bias is accounted for, trophic position may be substantially underestimated (Dale et al. 2011; Nielsen et al. 2015).

Among source amino acids in control sharks, we found a continuum of $\Delta^{15}\text{N}$ values, similar to previous studies (McMahon and McCarthy 2016). Only the discrimination factor for phenylalanine met our expectation of not differing from zero, emphasizing its role as a true

source amino acid that tracks the $\delta^{15}\text{N}$ value of producers at the base of the food web. The other source amino acids (lysine, tyrosine) had discrimination factors that differed from zero and overlapped with at least one trophic amino acid. Glycine also had a positive discrimination factor, and for control sharks serine exhibited a surprisingly negative discrimination factor; this reinforces the recent conclusion that these two amino acids should not be classified as “source” because of their highly-variable discrimination factors in different systems (McMahon and McCarthy 2016). In combination, the patterns in our data suggest that studies of elasmobranchs should consider phenylalanine as the most reliable source amino acid, although this conclusion should be tested in other elasmobranch species.

Conclusion. Elasmobranchs have important ecological roles and can structure marine communities (Young et al. 2015; Bird et al. 2018). Stable isotope analysis can be a powerful tool for assessing these roles (Hussey et al. 2011; Shiffman et al. 2012) but an understanding of how the unique physiology of elasmobranchs influences tissue isotopic patterns is needed to better interpret data collected from wild populations. Overall, we observed higher than expected $\Delta^{13}\text{C}$ values for essential amino acids (possibly because of microbial contributions), lower than expected $\Delta^{15}\text{N}$ values for trophic amino acids (likely because of urea recycling), and evidence that turnover in muscle is slow enough such that shark diet likely cannot be resolved at sub-annual time scales, an issue that can be exacerbated by switching among diet items which differ substantially in $\delta^{13}\text{C}$ or $\delta^{15}\text{N}$ values. Future studies of free-ranging elasmobranchs should account for these influences when inferring diet composition, trophic level, and habitat use. When these questions are addressed with amino acid isotope data, researchers can include sensitivity analyses of how their conclusions vary after adjusting discrimination factors based on

our results. This will help illustrate the capabilities and limitations of isotope-based approaches in ecology.

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Table 1. Amino acid composition (mole percent) of the muscle of leopard sharks (N = 3) and two prey items, squid (N = 1) and tilapia (N = 1). Amino acids are classified for carbon as essential (E) or non-essential (N), and for nitrogen as source (S) or trophic (T). For nitrogen, threonine is considered neither source nor trophic, and serine and glycine can act as either source or trophic depending upon the organism and ecosystem. Shark data are reproduced from Kim and Koch (2012).

<u>Classification</u>	<u>Amino Acid</u>	<u>Shark</u>	<u>Squid</u>	<u>Tilapia</u>
E	Threonine	6.0	7.0	8.0
ES	Lysine	11.1	7.9	5.1
ES	Phenylalanine	4.0	4.2	4.5
ET	Isoleucine	4.1	4.7	4.0
ET	Valine	6.4	7.3	8.7
ET	Leucine	9.3	10.1	9.3
NT	Aspartic Acid	13.4	13.5	13.3
NT	Glutamic Acid	10.2	9.5	7.8
NT	Alanine	10.4	12.3	10.1
NT	Proline	5.3	4.7	6.5
NS	Tyrosine	1.7	1.6	2.7
NT/S	Serine	6.2	7.0	10.2
NT/S	Glycine	12.0	10.2	9.8

Table 2. Test statistics for linear regression models in which amino acid imbalance between leopard sharks and their diet (measured as the difference in mole percent) was the predictor of stable isotope discrimination factors ($\Delta^{13}\text{C}$ and $\Delta^{15}\text{N}$) between leopard sharks and their diet. Two groups of sharks were evaluated: control (fed a constant diet of squid for 1250 days) and experimental (diet switched to tilapia on day 565). One model exhibited a significant relationship as indicated by an asterisk. However, after removal of a single data point (serine) on the basis of a high Cook's Distance score, the relationship was not significant.

Predictor	Response	Group	Amino acids	N	<i>P</i>	F	<i>R</i> ²
Amino acid imbalance	$\Delta^{13}\text{C}$	Control	All	12	0.95	0.00	<0.01
		Control	Non-essential	6	0.66	0.22	<0.01
	$\Delta^{15}\text{N}$	Control	All	12	0.52	0.44	<0.01
		Control	Trophic	6	0.93	0.01	<0.01
	$\Delta^{13}\text{C}$	Experimental	All	12	0.62	0.25	<0.01
		Experimental	Non-essential*	6	0.05	7.73	0.57
		Experimental	Non-essential (no serine)	5	0.41	0.93	<0.01
		Experimental	Trophic	6	0.96	0.00	<0.01
	$\Delta^{15}\text{N}$	Experimental	All	12	0.66	0.21	<0.01
		Experimental	Trophic	6	0.96	0.00	<0.01

687 Table 3. The percent of carbon and nitrogen estimated to have turned over in pools of individual
 688 amino acids in the muscle of six leopard sharks after a 1250-day experiment. Sharks were fed
 689 squid (days 0–565) then tilapia (days 566–1250).

690

Element	Amino acid	Turnover (%)
C	Glycine	50
	Phenylalanine	36
	Lysine	52
	Isoleucine	64
N	Alanine	34
	Aspartic acid	30
	Glutamic acid	29
	Isoleucine	31
	Leucine	32
	Valine	42
	Serine	17
	Threonine	47

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Figure 1. Conceptual figure illustrating the effect of incomplete turnover on the appearance of carbon isotope discrimination factors ($\Delta^{13}\text{C}_{\text{consumer} - \text{diet}}$). The concepts apply to other isotopes as well. a–c) Each panel represents the $\delta^{13}\text{C}$ values of an amino acid (X, Y, or Z) measured in the tissue of a consumer (black line) that switched from an old diet to a new diet (dashed gray lines) on day 0. Each amino acid had the same turnover rate and if the consumer was in a steady state with its diet a $\Delta^{13}\text{C}$ value of 2‰. The y-axis is identical across panels. The differences in $\delta^{13}\text{C}$ values between the old diet and new diet cause $\Delta^{13}\text{C}$ to differ between days 100 (Δ_{100}) and 500 (Δ_{500}). d) On day 100, amino acid X already had a $\Delta^{13}\text{C}$ value that was close to 2‰ because the old diet and new diet were similar in $\delta^{13}\text{C}$ values. However, for amino acid Z, $\Delta^{13}\text{C}$ was much larger than 2‰ because the old diet and new diet differed substantially in $\delta^{13}\text{C}$ values. e) On day 500, when turnover was nearly complete, each amino acid had a $\Delta^{13}\text{C}$ value of about 2‰.

Figure 2. Carbon isotope values of individual amino acids in leopard shark muscle and in diet items of the sharks. Box plots indicate median (solid line) and 25th, 10th, and 5th percentiles. a) $\delta^{13}\text{C}$ values of control sharks (fed squid for 1250 days; N = 3), experimental sharks (switched to a diet of tilapia on day 565; N = 6), and their respective diet items (squid, N = 3; tilapia, N = 2). b) Discrimination factors ($\Delta^{13}\text{C}$) for control and experimental sharks. Asterisks above boxes indicate *P* values for a one-tailed t-test of whether discrimination factors differed from zero (test statistics listed in Table S2). Brackets and asterisks below boxes indicate *P* values for a two-tailed t-test of whether control and experimental sharks differed (test statistics listed in Table S3).

Figure 3. Variation in consumer-diet isotope discrimination factors (Δ) for the a) carbon and b) nitrogen of individual amino acids in muscle tissue of leopard sharks in a control group (consumed squid for 1250 days) or experimental group (switched to tilapia on day 565). The x-axes represent the difference between the mean isotopic value of each amino acid for control (squid) diet and the experimental (tilapia) diet for a) $\delta^{13}\text{C}$ and b) $\delta^{15}\text{N}$. The y-axes represent the difference in the mean discrimination factor of each amino acid between experimental and control individuals. The solid line represents a linear regression (dashed line is 95% CI). Once an animal reaches a steady state with a new diet the slope in each panel should be zero, if the new diet has similar protein quality and content as the old diet.

Figure 4. Nitrogen isotope values of individual amino acids in leopard shark muscle and in diet items of the sharks. Box plots indicate median (solid line) and 25th, 10th, and 5th percentiles. a) $\delta^{15}\text{N}$ of control sharks (fed squid for 1250 days; N = 3), experimental sharks (switched to a diet of tilapia on day 565; N = 6), and their respective diet items (squid, N = 3; tilapia, N = 2). Note that the box for tyrosine in the experimental diet is difficult to see because it is small and nearly identical to the line which forms the bottom of the box for experimental sharks (i.e., 10th percentile for the experimental sharks). b) Discrimination factors ($\Delta^{15}\text{N}$) for control and experimental sharks. Asterisks above boxes indicate *P* values for a one-tailed t-test of whether discrimination factors differed from zero (test statistics listed in Table S2). Brackets and asterisks below boxes indicate *P* values for a two-tailed t-test of whether control and experimental sharks differed (test statistics listed in Table S3).

Figure 1.

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751 Figure 4.

Amino acid isotope discrimination factors for a carnivore: physiological insights from leopard sharks and their diet

Supplementary Materials

Authors

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Table S1. Bulk carbon and nitrogen isotope measurements of muscle samples from captive leopard sharks.

Shark ID	Experimental day	$\delta^{13}\text{C}$	$\delta^{15}\text{N}$
BL	28	-16.8	19.6
BL	42	-17.9	18.2
BL	61	-17.5	18.5
BL	87	-17.3	17.6
BL	98	-17.0	17.9
BL	112	-17.2	17.7
BL	126	-17.2	18.0
BL	140	-17.4	17.0
BL	154	-17.4	18.2
BL	168	-16.8	17.0
BL	182	-16.1	14.3
BL	196	-16.9	15.5
BL	210	-18.3	19.6
BL	239	-16.7	17.9
BL	273	-16.5	15.5
BL	308	-17.2	16.9
BL	329	-17.0	16.7
BL	400	-17.0	17.0
BL	455	-16.9	16.1
BL	475	-16.4	15.4
BL	496	-16.4	15.9
BL	591	-17.8	15.8
BL	615	-17.5	16.5
BL	633	-18.2	15.2
BL	654	-18.3	15.5
BL	665	-20.3	13.1
BL	696	-18.4	14.8
BL	749	-19.5	14.0
BL	791	-18.8	14.8
BL	833	-18.7	15.3
BL	875	-19.1	15.3
BL	917	-19.4	15.1
BL	959	-19.7	14.4
BL	997	-21.1	12.9
BL	1039	-19.7	14.4
BL	1090	-20.4	14.6
BL	1131	-21.4	12.5

Shark ID	Experimental day	$\delta^{13}\text{C}$	$\delta^{15}\text{N}$
BL	1153	-20.8	13.8
BL	1174	-21.3	12.4
BS	28	-12.2	5.6
BS	42	-18.3	19.4
BS	61	-16.1	19.1
BS	94	-17.4	18.1
BS	98	-17.5	18.5
BS	112	-17.7	18.1
BS	126	-17.7	18.5
BS	140	-17.6	17.9
BS	168	-42.1	17.3
BS	182	-17.6	16.9
BS	196	-17.2	16.3
BS	210	-17.4	17.8
BS	239	-16.9	16.9
BS	273	-17.6	16.5
BS	308	-17.6	16.9
BS	329	-17.0	16.5
BS	400	-17.0	16.2
BS	455	-17.0	15.9
BS	475	-16.9	15.4
BS	496	-16.5	15.9
BS	591	-17.1	16.0
BS	615	-17.5	16.3
BS	633	-18.2	15.9
BS	654	-19.0	14.6
BS	665	-18.0	16.5
BS	696	-18.6	15.1
BS	749	-18.6	15.1
BS	791	-19.4	14.7
BS	833	-19.5	14.0
BS	875	-19.1	14.9
BS	917	-20.6	13.0
BS	959	-19.8	14.4
BS	997	-21.0	12.1
BS	1039	-21.2	12.5
BS	1090	-21.7	13.1
BS	1131	-21.2	12.6
BS	1153	-21.0	13.3
BS	1174	-20.7	13.3

Shark ID	Experimental day	$\delta^{13}\text{C}$	$\delta^{15}\text{N}$
CS	28	-16.8	19.3
CS	42	-18.1	17.9
CS	61	-17.1	19.7
CS	87	-14.9	20.3
CS	98	-16.5	19.4
CS	112	-17.0	19.2
CS	140	-16.5	18.3
CS	154	-16.3	18.1
CS	154	-16.8	18.0
CS	168	-16.2	18.4
CS	182	-16.7	18.0
CS	196	-16.6	16.8
CS	210	-18.0	18.7
CS	239	-16.3	17.5
CS	273	-15.9	13.7
CS	308	-16.5	17.0
CS	329	-16.6	16.3
CS	329	-16.7	16.3
CS	400	-15.7	17.3
CS	455	-16.6	16.0
CS	475	-16.6	16.5
CS	496	-16.6	15.8
CS	591	-16.8	16.6
CS	615	-16.6	17.4
CS	633	-16.7	17.4
CS	654	-16.4	15.9
CS	665	-16.8	17.0
CS	696	-16.7	17.5
CS	749	-17.1	17.6
CS	791	-16.7	17.3
CS	833	-16.8	17.2
CS	875	-16.8	16.9
CS	917	-16.7	16.5
CS	959	-16.3	17.4
CS	997	-16.5	17.1
CS	1039	-16.7	17.2
CS	1090	-16.4	17.1
CS	1131	-16.7	16.5
DL	54	-16.8	17.7
DL	94	-16.6	18.5

Shark ID	Experimental day	$\delta^{13}\text{C}$	$\delta^{15}\text{N}$
DL	94	-16.8	18.4
DL	105	-16.4	17.7
DL	119	-16.9	17.1
DL	133	-16.8	18.0
DL	161	-16.7	17.3
DL	175	-16.6	18.7
DL	189	-16.5	16.6
DL	203	-16.6	14.5
DL	231	-15.9	17.9
DL	267	-16.3	16.9
DL	301	-16.4	17.8
DL	357	-16.9	16.5
DL	392	-16.7	16.9
DL	420	-14.2	18.1
DL	435	-17.1	17.2
DL	475	-15.9	16.3
DL	496	-16.6	16.7
DL	591	-16.9	16.1
DL	615	-17.8	15.2
DL	633	-18.2	14.9
DL	654	-18.2	14.9
DL	665	-18.1	15.6
DL	696	-18.2	15.3
DL	749	-18.0	16.0
DL	791	-19.6	13.8
DL	875	-17.9	15.7
DL	917	-18.2	15.3
DL	959	-18.9	14.8
DL	997	-20.0	13.3
DL	1039	-20.2	13.8
DL	1090	-19.9	14.2
DL	1131	-19.4	14.4
DL	1153	-19.9	13.8
DL	1174	-19.5	14.1
DS	54	-16.1	21.2
DS	94	-17.1	21.5
DS	105	-17.1	21.1
DS	119	-17.4	20.5
DS	126	-17.3	19.0
DS	133	-17.5	20.9

Shark ID	Experimental day	$\delta^{13}\text{C}$	$\delta^{15}\text{N}$
DS	146	-17.4	20.5
DS	161	-17.3	19.3
DS	175	-16.4	17.3
DS	189	-16.6	17.7
DS	203	-17.1	18.4
DS	231	-16.7	18.0
DS	267	-16.5	18.7
DS	301	-16.5	15.7
DS	322	-16.7	16.2
DS	357	-17.0	18.4
DS	392	-16.6	18.0
DS	420	-15.6	16.2
DS	435	-17.0	21.5
DS	475	-16.7	15.7
DS	496	-16.4	15.6
DS	591	-16.8	17.7
DS	615	-17.2	17.1
DS	633	-17.3	17.1
DS	654	-17.8	16.4
DS	665	-17.8	17.2
DS	696	-18.3	15.4
DS	749	-18.1	16.5
DS	791	-19.8	13.6
DS	833	-18.5	17.6
DS	875	-19.5	14.5
DS	917	-20.1	13.7
DS	959	-19.8	13.6
DS	997	-20.3	13.3
DS	1039	-20.4	13.5
DS	1090	-20.4	13.7
DS	1131	-20.6	13.9
DS	1153	-21.2	12.8
DS	1174	-20.5	13.6
EL	54	-17.8	19.2
EL	94	-16.6	19.3
EL	119	-17.4	17.4
EL	161	-16.9	17.5
EL	175	-17.6	18.7
EL	189	-17.2	16.2
EL	203	-17.1	16.1

Shark ID	Experimental day	$\delta^{13}\text{C}$	$\delta^{15}\text{N}$
EL	231	-16.6	16.7
EL	267	-15.9	18.2
EL	301	-16.8	15.8
EL	322	-16.6	15.9
EL	357	-17.2	18.4
EL	392	-16.7	16.1
EL	420	-16.2	15.7
EL	435	-17.6	18.4
EL	475	-15.8	16.6
EL	503	-16.6	16.1
EL	591	-16.8	15.8
EL	615	-17.1	16.1
EL	633	-17.5	15.3
EL	654	-17.6	15.2
EL	665	-18.0	15.3
EL	696	-17.8	15.1
EL	749	-17.9	15.4
EL	791	-17.8	15.2
EL	833	-18.3	14.6
EL	875	-16.6	16.7
EL	917	-18.0	15.3
EL	959	-19.3	13.5
EL	997	-19.2	13.8
EL	1039	-20.7	12.7
EL	1090	-19.5	14.3
EL	1131	-19.7	13.5
EL	1153	-19.1	14.2
EL	1174	-19.9	13.1
ES	54	-16.3	17.4
ES	94	-16.1	17.6
ES	105	-16.3	17.2
ES	119	-16.4	16.2
ES	133	-16.6	17.4
ES	146	-16.4	17.4
ES	161	-16.1	16.7
ES	175	-16.8	19.8
ES	189	-16.2	16.1
ES	203	-16.1	15.9
ES	231	-36.5	14.0
ES	267	-16.2	16.1

Shark ID	Experimental day	$\delta^{13}\text{C}$	$\delta^{15}\text{N}$
ES	301	-17.0	16.4
ES	322	-16.7	16.4
ES	357	-16.7	16.1
ES	392	-16.3	16.4
ES	420	-16.4	15.7
ES	435	-16.6	16.4
ES	475	-16.5	16.0
ES	503	-16.2	15.3
ES	591	-16.8	16.1
ES	615	-17.4	15.5
ES	633	-17.5	15.2
ES	654	-17.2	15.9
ES	665	-17.4	16.0
ES	696	-17.4	15.8
ES	749	-17.5	15.5
ES	791	-18.4	15.2
ES	833	-18.0	15.2
ES	875	-18.0	15.7
ES	917	-17.5	15.3
ES	959	-18.5	14.4
ES	997	-18.7	14.3
ES	1039	-19.2	14.0
ES	1090	-19.7	14.1
ES	1131	-19.2	14.1
ES	1153	-20.0	13.2
ES	1174	-20.3	12.8
FL	54	-17.2	19.6
FL	94	-17.3	19.8
FL	105	-17.1	17.8
FL	133	-17.7	20.4
FL	161	-17.5	18.3
FL	175	-17.4	18.2
FL	189	-17.5	17.5
FL	203	-17.3	16.7
FL	231	-17.7	18.2
FL	267	-16.4	15.4
FL	301	-16.9	16.5
FL	322	-16.6	15.9
FL	357	-17.2	16.9
FL	392	-16.9	16.4

Shark ID	Experimental day	$\delta^{13}\text{C}$	$\delta^{15}\text{N}$
FL	420	-16.1	15.6
FL	435	-17.8	19.8
FL	475	-16.6	16.4
FL	503	-16.4	16.0
FL	591	-16.8	17.1
FL	615	-16.8	17.3
FL	633	-17.0	17.4
FL	654	-16.9	17.2
FL	665	-16.9	17.6
FL	696	-16.6	18.0
FL	749	-16.9	17.9
FL	791	-16.6	17.0
FL	833	-16.7	16.7
FL	875	-16.8	17.6
FL	917	-16.7	17.2
FL	959	-16.7	17.6
FL	997	-16.7	17.5
FL	1039	-16.6	17.3
FS	54	-17.4	19.0
FS	94	-17.4	19.6
FS	119	-17.2	18.4
FS	133	-17.3	18.6
FS	161	-17.0	17.9
FS	175	-16.9	17.9
FS	189	-16.4	17.1
FS	203	-17.2	16.6
FS	231	-16.8	16.6
FS	267	-16.2	16.2
FS	301	-17.0	16.4
FS	322	-16.4	15.6
FS	357	-17.2	17.2
FS	392	-16.1	16.3
FS	420	-16.4	15.9
FS	435	-17.1	18.8
FS	475	-17.1	16.9
FS	503	-16.2	15.6
FS	591	-16.7	16.4
FS	615	-16.6	16.6
FS	633	-16.8	17.0
FS	654	-17.0	17.3

Shark ID	Experimental day	$\delta^{13}\text{C}$	$\delta^{15}\text{N}$
FS	665	-16.9	17.2
FS	696	-17.0	16.8
FS	749	-16.9	16.8
FS	791	-16.6	16.9
FS	833	-17.0	16.6
FS	875	-15.9	17.0
FS	917	-16.7	17.2
FS	959	-16.5	16.8
FS	997	-16.4	16.9
FS	1039	-16.5	17.0
FS	1131	-16.7	16.4

Table S2. Statistics (DF = degrees of freedom) for one-tailed t-tests of whether stable isotope discrimination factors ($\Delta_{\text{shark muscle-diet}}$) for captive leopard sharks differed from 0‰. Sharks were either fed squid for 1250 days (control group), or squid then tilapia as of day 565 (experimental group).

	Amino acid	Group	<i>P</i>	<i>t</i>	DF
$\Delta^{13}\text{C}$	Alanine	Control	0.049	2.956	2
	Alanine	Experimental	<0.001	9.902	5
	Glycine	Control	0.041	3.275	2
	Glycine	Experimental	<0.001	11.539	5
	Threonine	Control	0.123	1.619	2
	Threonine	Experimental	0.142	1.021	5
	Serine	Control	0.316	-0.561	2
	Serine	Experimental	0.294	0.579	5
	Valine	Control	0.026	4.175	2
	Valine	Experimental	<0.001	10.974	5
	Leucine	Control	0.019	4.942	2
	Leucine	Experimental	<0.001	13.358	5
	Isoleucine	Control	0.007	8.325	2
	Isoleucine	Experimental	<0.001	7.510	5
	Aspartic acid	Control	0.013	5.984	2
	Aspartic acid	Experimental	<0.001	15.745	5
	Glutamic acid	Control	0.008	7.879	2
	Glutamic acid	Experimental	<0.001	12.513	5
	Phenylalanine	Control	0.078	2.228	2
	Phenylalanine	Experimental	<0.001	7.554	5
$\Delta^{15}\text{N}$	Tyrosine	Control	0.040	3.318	2
	Tyrosine	Experimental	<0.001	11.882	5
	Lysine	Control	0.114	1.721	2
	Lysine	Experimental	<0.001	8.995	5
	Alanine	Control	0.026	4.201	2
	Alanine	Experimental	<0.001	19.247	5
	Glycine	Control	0.045	3.120	2
	Glycine	Experimental	0.020	2.752	5
	Threonine	Control	0.012	-6.426	2
	Threonine	Experimental	<0.001	-37.709	5
	Serine	Control	0.017	-5.355	2
	Serine	Experimental	0.038	2.225	5
	Valine	Control	0.004	10.842	2
	Valine	Experimental	<0.001	21.664	5
	Leucine	Control	0.005	10.033	2
	Leucine	Experimental	0.005	10.033	5
	Isoleucine	Control	0.001	20.349	2
	Isoleucine	Experimental	<0.001	30.074	5
	Aspartic acid	Control	0.030	3.869	2
	Aspartic acid	Experimental	<0.001	21.011	5

	Amino acid	Group	<i>P</i>	<i>t</i>	DF
$\Delta^{15}\text{N}$	Glutamic acid	Control	0.007	8.668	2
	Glutamic acid	Experimental	<0.001	19.147	4
	Phenylalanine	Control	0.171	1.236	2
	Phenylalanine	Experimental	0.484	0.414	5
	Tyrosine	Control	0.010	6.937	2
	Tyrosine	Experimental	0.021	2.699	5
	Lysine	Control	0.014	5.863	2
	Lysine	Experimental	0.002	4.816	5

Table S3. Statistics (DF = degrees of freedom) for two-tailed t-tests of whether stable isotope discrimination factors ($\Delta_{\text{shark muscle-diet}}$) for captive leopard sharks differed between a control group and experimental group. The control group was fed squid for 1250 days and the experimental group was fed squid then tilapia as of day 565.

	Amino acid	P	t	DF
$\Delta^{13}\text{C}$	Alanine	0.056	-2.289	7
	Glycine	0.015	-3.190	7
	Threonine	0.971	-0.038	7
	Serine	0.535	-0.652	7
	Valine	0.381 ^a	11.000 ^a	NA ^a
	Leucine	0.149	-1.619	7
	Isoleucine	0.045	-2.431	7
	Aspartic acid	0.753	0.327	7
	Glutamic acid	0.488	0.731	7
	Phenylalanine	0.003	-3.844	7
	Tyrosine	0.069	-2.144	7
	Lysine	0.008	-3.691	7
$\Delta^{15}\text{N}$	Alanine	<0.001	-9.046	7
	Glycine	0.496	0.719	7
	Threonine	0.001	5.257	7
	Serine	0.002	-4.672	7
	Valine	<0.001	-6.291	7
	Leucine	<0.001	-14.119	7
	Isoleucine	<0.001	-10.691	7
	Aspartic acid	<0.001	-7.663	7
	Glutamic acid	<0.001	-9.186	6
	Phenylalanine	0.397	0.902	7
	Tyrosine	0.890	-0.143	7
	Lysine	.107	1.849	7

^aStatistics from Mann-Whitney rank sum test because data failed Shapiro-Wilk test of normality.

Figure S1. Data from six leopard sharks (individuals identified by a two-letter code) used in this captive feeding study. Measurements include body length (solid black line and circles) and stable isotope values of bulk muscle tissue ($\delta^{13}\text{C}$ values are black dashed line and circles, $\delta^{15}\text{N}$ values are dashed gray line and circles). Sharks were switched from a squid diet to a tilapia diet on 01-August-2007. Also shown is temperature (thin black line) of seawater in Monterey Bay, California, USA, which was circulated in the shark tanks. Water temperature was measured in the bay at 2 meters below mean lower low water (MLLW) by the National Oceanic and Atmospheric Administration (sensor 9413450, data from <https://tidesandcurrents.noaa.gov/>). Shark data are reproduced from Kim et al. (2012).

Reference

Kim SL, Martínez del Río C, Casper D, Koch PL. 2012. Isotopic incorporation rates for shark tissues from a long-term captive feeding study. *Journal of Experimental Biology* 215:2495-2500.

Figure S2. Consumer-diet discrimination factors for stable nitrogen isotopes ($\Delta^{15}\text{N}$) of leopard sharks consuming squid, as measured in this study and as measured in Hoen et al. (2014). Box plots indicate median (solid line) and 25th, 10th, and 5th percentiles.

Reference

Hoen DK, Kim SL, Hussey NE, Wallsgrove NJ, Drazen JC, Popp BN. 2014. Amino acid ^{15}N enrichment factors of four large carnivorous fishes. *Journal of Experimental Marine Biology and Ecology* 453:76-83.