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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 ($\Delta^{15}N$) than most previous consumer studies, potentially because of urea recycling. Control sharks also had large carbon isotope discrimination factors ($\Delta^{13}C$) for three essential amino acids, suggesting microbial contributions or fractionation upon assimilation. Compared to controls, experimental sharks exhibited higher $\Delta^{13}C$ values for four amino acids and $\Delta^{15}N$ values for seven amino acids, corresponding with differences between diets in $\delta^{13}C$ and $\delta^{15}N$ 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 $\delta^{13}C$ and $\delta^{15}N$ 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 ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) 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 $\delta^{13}C$ and $\delta^{15}N$ 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 $\delta^{13}C$ and $\delta^{15}N$ values between consumers and their diet (e.g., $\Delta^{15}N = \delta^{15}N_{consumer} - \delta^{15}N_{diet}$). 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 Rio 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 $\delta^{13}C$ 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 $\delta^{15}N$ 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 $\delta^{15}N$ 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 discimination factors, although contradictory trends have been reported with high protein correlating with both increased (Kelly and Martínez del Rio 2010) and

87 decreased discimination (Hughes et al. 2018). Thus, discrimination factors for bulk tissue and
88 amino acids of consumers can be influenced by their diet, physiology, and trophic level.
89 Discrimination factors are often assessed with controlled feeding experiments using
90 captive animals. Such experiments must also consider isotopic turnover rate, which is the pace of
91 incorporation of dietary carbon and nitrogen into consumer biomass during tissue addition (i.e.,
92 accretion during growth) or replacement (i.e., maintenance). Turnover rate is influenced by
93 metabolic rate and body size, among other factors (Martínez del Rio and Carleton 2012; Vander
94 Zanden et al. 2015). Most elasmobranchs are ectotherms, which tend to exhibit slow turnover
95 rates (Vander Zanden et al. 2015), and sharks can take over a year for their bulk muscle tissue to
96 reach a steady state with the isotopic composition of a new diet (Malpica-Cruz et al. 2012; Kim
97 et al. 2012b). Importantly, incomplete turnover can cause discrimination factors to appear
98 different than they would for an animal that has reached steady state (Fig. 1).
99 Here, we use a long-term feeding study to evaluate variations in carbon and nitrogen
100 isotope discrimination factors for amino acids in muscle tissue from leopard sharks (*Triakis*
101 *semifasciata*), an abundant predator in Pacific coastal waters of North America. Nine captive
102 leopard sharks were fed squid (*Loligo opalescens*) for 565 days then divided into control and
103 experimental groups (Kim et al. 2012a, b). For the subsequent 685 days, control sharks (N = 3)
104 continued eating squid while experimental sharks (N = 6) were fed tilapia (*Oreochromis* sp.).
105 Bulk tissue analysis of muscle samples collected at the end of the experiment indicated that $\delta^{13}C$
106 and $\delta^{15}N$ of both control and experimental sharks had reached steady states with their respective
107 diets (Kim et al. 2012a, b), although bulk $\Delta^{13}C$ and $\Delta^{15}N$ differed between diets. Using the same
108 set of muscle samples, here we test the fundamental assumptions that isotopic discrimination
109 factors are 1) near zero for essential amino acids ($\Delta^{13}C$) and source amino acids ($\Delta^{15}N$), and 2)

larger than zero for non-essential amino acids ($\Delta^{13}C$) and trophic amino acids ($\Delta^{15}N$). 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 $\delta^{13}C$ and $\delta^{15}N$ 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 N_2 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 CO_2 or reduced to N_2 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 $\delta^{13}C$ values (with a standard every fifth injection) and three injections for $\delta^{15}N$ values (with a standard every fourth injection). SD for multiple injections of the same sample averaged 0.16‰ (range 0.00–0.62) for $\delta^{13}C$ and 0.55‰ (0.05–1.92) for $\delta^{15}N$ 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 $\delta^{13}C$

and 0.81‰ (0.00–1.69) for $\delta^{15}N$ 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, $\delta^{13}C$ values were corrected as follows:

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

Here, $\delta^{13}C_{sample.underiv}$ is the final, calculated value of the amino acid; $\delta^{13}C_{sample.deriv}$ is the measured value of the derivatized amino acid; $\delta^{13}C_{standard.underiv}$ 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 $\delta^{15}N$ values was less complex because derivatization does not add exogenous nitrogen:

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

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 ($\Delta^{15}N_{shark\ muscle\ -\ diet}$ and $\Delta^{13}C_{shark\ muscle\ -\ diet}$) 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, $\Delta^{13}C$ values

should be larger for non-essential than for essential amino acids and $\Delta^{15}N$ 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 $\Delta^{13}C$ values; and using only trophic amino acids for $\Delta^{15}N$ values. Linear regressions were used to assess the relationship between 1) the difference between diets in $\delta^{13}C$ or $\delta^{15}N$ values, and 2) the difference in $\Delta^{13}C$ or $\Delta^{15}N$ 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 (±1% SD) and the tilapia diet was 12% (±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 $\Delta^{13}C$ 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),

223 differed for $\delta^{13}C$ values (squid: -18.5±0.5‰ SD, tilapia: -23.2±0.9‰) and $\delta^{15}N$ values (squid:
224 13.3±0.7‰, tilapia:7.9±0.4‰).
225
226 *Carbon isotope discrimination.* Amino acids varied in $\delta^{13}C$ values for squid, tilapia, and shark
227 muscle (Fig. 2a). Both experimental and control sharks exhibited a wide range of amino acid
228 discrimination factors (Fig. 2b), although several $\Delta^{13}C$ patterns were similar between these two
229 groups. Among non-essential amino acids, both experimental and control sharks exhibited a
230 discrimination factor for serine that did not differ from zero. In contrast, both groups exhibited
231 large discrimination factors for the ketogenic amino acids (alanine, aspartic acid, glutamic acid,
232 tyrosine). Among essential amino acids, both experimental and control sharks showed no
233 discrimination for threonine but high discrimination for leucine and valine. Discrimination
234 factors for other amino acids differed between groups. One non-essential (glycine) and three
235 essential (phenylalanine, lysine, and isoleucine) amino acids had larger discrimination factors for
236 experimental sharks than for control individuals (Fig. 2b). The differences in $\Delta^{13}C$ values
237 between experimental and control sharks correlated with the $\delta^{13}C$ differences between their
238 respective diet items (tilapia and squid; Fig. 3a). Assuming that the control sharks were in a
239 steady state with their diet (see Discussion) and based on the similar nutrition of the two diets, it
240 is expected that the experimental sharks would eventually exhibit the same amino acid $\Delta^{13}C$
241 values as control sharks. Thus, amino acids that did not differ in $\Delta^{13}C$ between control and
242 experimental sharks either had similar $\delta^{13}C$ values between diets or had turned over ~100% of
243 their carbon pool in the experimental sharks. In contrast, the amino acids that differed in $\Delta^{13}C$
244 between control and experimental sharks were calculated to have turned over 36–64% of their
245 carbon pool (Table 3).

246

247 *Nitrogen isotope discrimination.* Amino acids varied in $\delta^{15}N$ values for squid, tilapia, and shark
248 muscle (Fig. 4a). For most source amino acids, $\delta^{15}N$ values were similar for squid, tilapia,
249 experimental shark muscle, and control shark muscle, resulting in relatively small amino acid
250 discrimination factors between diet and consumer (Fig. 4b). For both experimental and control
251 sharks, discrimination did not differ from zero for phenylalanine and median values were <3‰
252 for lysine and tyrosine. Between groups, median $\Delta^{15}N$ values were similar for glycine (1–2‰)
253 and dissimilar for serine (2‰ for experimental, -5‰ for control). Discrimination factors for
254 threonine and for trophic amino acids were greater for the experimental group than for the
255 control group. For experimental sharks, trophic amino acids had a mean discrimination factor of
256 9.8‰ (±1.6 SD), while the mean for control individuals was 3.8‰ (±1.4). The discrimination
257 factor for threonine was less for experimental sharks (median of -11‰) than for control sharks
258 (median of -8‰). The differences in $\Delta^{15}N$ values between experimental and control individuals
259 correlated with $\delta^{15}N$ differences between their respective diet items (tilapia and squid; Fig. 3b).
260 The Cook's Distance score indicated that threonine was a potential outlier in this relationship;
261 however, regression statistics were almost unchanged after its removal ($P < 0.01$, $R^2 = 0.95$).
262 Similar to carbon, it is expected that the experimental sharks would eventually exhibit the same
263 amino acid $\Delta^{15}N$ values as control sharks. Thus, amino acids that did not differ in $\Delta^{15}N$ between
264 control and experimental sharks either had similar $\delta^{15}N$ values between diets or had turned over
265 ~100% of their nitrogen pool in the experimental sharks. In contrast, the amino acids that
266 differed in $\Delta^{15}N$ between control and experimental sharks were calculated to have turned over
267 17–47% of their nitrogen pool (Table 3).
268

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, $\Delta^{13}C$ and $\Delta^{15}N$ 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 $\Delta^{13}C$ 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

292 incomplete turnover for some amino acids in the experimental treatment, as discussed in detail
293 below.
294
295 *Experimental sharks.* Compared to control sharks, experimental individuals had greater $\Delta^{13}C$
296 values for one non-essential (glycine) and three essential amino acids (Fig. 2; phenylalanine,
297 lysine, isoleucine) and greater $\Delta^{15}N$ values for all six trophic amino acids (alanine, asx, glx,
298 isoleucine, leucine, valine) and threonine (Fig. 4). These differences in discrimination factors
299 could be caused by amino acids in the muscle of experimental sharks having not yet reached a
300 steady state with their new diet. This explanation is supported by the facts that 1) the new diet
301 was lower in $\delta^{13}C$ and $\delta^{15}N$, which would lead to the observed direction of change in amino acid
302 discrimination factors, and 2) the magnitude of difference in $\delta^{13}C$ and $\delta^{15}N$ values between diets
303 predicted the amount by which the amino acid discrimination factor increased after the diet
304 switch. Larger offsets between diet and consumer $\delta^{13}C$ or $\delta^{15}N$ values make it easier to discern a
305 lack of steady state conditions, whereas smaller offsets (especially those that are similar in
306 magnitude to analytical uncertainty) can create a perception of steady state when it has not yet
307 been achieved (Fig. 1). For example, aspartic acid was similar in $\delta^{13}C$ values between diets: -
308 23‰ for squid and -26‰ for tilapia. Thus, after the switch from squid to tilapia, the aspartic acid
309 of the experimental shark muscle only needed to decline by 3‰ to reach a new steady state, a
310 relatively small difference considering our analytical precision (0.2–0.8‰). By the end of the
311 experiment, aspartic acid appeared to have reached a steady state because its discrimination
312 factor in experimental sharks was nearly identical to that in control sharks. In contrast, the diets
313 had very different glycine $\delta^{13}C$ values (-6‰ for squid and -16‰ for tilapia), and thus this amino
314 acid had to decline by 10‰ in shark muscle to reach a new steady state. As a result, by the end

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

318 The variable magnitude of the required shift in isotopic values after the diet switch can
319 also contribute to dissociation between carbon and nitrogen dynamics. For example, the carbon
320 in the glycine of muscle in the experimental sharks appeared to have not yet reached a steady
321 state because of the large change required in $\delta^{13}C$ after the diet switch (10‰). However, the
322 required shift in glycine $\delta^{15}N$ was much smaller (1‰). Thus, as would be expected, the glycine
323 $\Delta^{15}N$ value for experimental individuals appeared to be very similar to that of control
324 individuals, giving the appearance that the nitrogen in glycine was close to a steady state with the
325 new (tilapia) diet.

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

384 consider that isotopic composition of muscle in sharks and other large, ectothermic marine
385 consumers likely cannot reveal seasonal shifts in diet or habitat use.
386
387 *Control sharks.* We believe that discrimination factors for individual amino acids in control
388 sharks were accurate and not influenced by incomplete incorporation of the diet, for several
389 reasons. First, by the time of sampling at the end of the experiment, control individuals had been
390 on a constant diet for 1250 days and had not exhibited substantial, directional change in bulk
391 tissue $\delta^{13}C$ or $\delta^{15}N$ values for >400 days (Kim et al. 2012a). Second, a recent review found that
392 the longest time interval reported for elasmobranch muscle to replace 95% of endogenous carbon
393 or nitrogen was 422 days (Galván et al. 2016). Although we propose that the appearance of
394 steady states may not always be reliable (as described in the previous section), the fact that
395 control sharks consumed the same diet for a period three times longer than the maximum
396 reported interval for 95% turnover makes it likely that they had reached a steady state.
397 Data from control sharks supported our prediction that discrimination factors would be
398 larger than zero for non-essential amino acids. The largest $\Delta^{13}C$ values in control sharks were for
399 aspartic acid and glutamic acid, suggesting extensive *de novo* synthesis. This result is consistent
400 with the roles of aspartic acid and glutamic acid as important metabolic intermediates in the
401 processing of nitrogen derived from amino acid catabolism, which is prevalent in
402 hypercarnivores such as the sharks in this study. In addition, aspartic acid and glutamic acid are
403 ketogenic and thus their synthetic pathways most immediately use other amino acids as a carbon
404 source, which would be plentiful for animals consuming a protein-rich diet. In contrast, control
405 sharks exhibited smaller $\Delta^{13}C$ values for the glycolytic amino acids (glycine, serine, alanine),
406 which are primarily synthesized using carbohydrates as a carbon source, which were relatively

limited in both diets. Glycine and alanine had positive $\Delta^{13}C$ values, suggesting some *de novo* synthesis, while serine was the only non-essential amino acid that did not support our predictions. Serine had a $\Delta^{13}C$ 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 $\delta^{13}C$ value of the remaining, assimilated amino acids would increase and the $\Delta^{13}C$ 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 $\Delta^{13}C$ 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 $\delta^{13}C$ values of consumer tissue.

In comparison to $\Delta^{13}C$ values, control sharks exhibited less variation in $\Delta^{15}N$ values (note that our $\Delta^{15}N$ 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 $\Delta^{15}N$ 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 $\Delta^{15}N$ 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 $\Delta^{15}N$ values were similar to those reported from controlled feeding experiments with other sharks and a carnivorous fish (opakapaka; *Pristipomoides filamentosus*; Hoen et al. 2014). Lower than expected $\Delta^{15}N$ values of trophic amino acids have also been predicted for free-ranging brown stingrays (*Dasyatic 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 $\Delta^{15}N$ 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 $\Delta^{13}C$ values are indicative of extensive *de novo* synthesis. For example, among non-essentials, aspartic acid and glutamic acid exhibited $\Delta^{13}C$ values of 9–16‰ but $\Delta^{15}N$ values of only 2–4‰.

We suggest that the relatively small $\Delta^{15}N$ 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

453 animals, catabolism of amino acids creates a pool of nitrogen, of which ^{14}N is selectively
454 incorporated into urea then excreted. The remaining nitrogen pool becomes relatively enriched in
455 ^{15}N and is used for synthesis of some endogenous amino acids, leading to large $\Delta^{15}N$ values (Lee
456 et al. 2012). However, sharks retain urea for use as a tissue osmolyte (Ballantyne 1997). This
457 process is so important that some sharks synthesize additional urea by converting ammonia from
458 surrounding seawater (Wood and Giacomin 2016). Sharks, like most vertebrates, likely lack the
459 enzymes for hydrolyzing urea and recycling its nitrogen but can host populations of bacteria
460 capable of urea hydrolysis (Stevens and Hume 1998). Indeed, such populations occur in shark
461 muscle (Grimes et al. 1985) and bacterially-mediated urea breakdown has been demonstrated in
462 shark liver tissue (Knight et al. 1988). Sharks have high rates of amino acid catabolism and urea
463 production, which would typically lead to elevated $\Delta^{15}N$ values if that urea was excreted; but
464 they retain it and it is highly feasible that sharks then rely on bacterial symbionts to break down
465 the urea and make the nitrogen therein available for re-use. The incorporation of ^{14}N recycled
466 from urea into newly-synthesized amino acids and endogenous tissue could explain the low $\Delta^{15}N$
467 values of trophic amino acids in sharks. This assimilation of microbially-produced amino acids
468 could also lead to the non-zero $\Delta^{13}C$ that we observed for some essential amino acids.
469 Researchers interpreting isotope data from free-ranging elasmobranchs should consider that
470 individuals may recycle urea and exhibit a $\Delta^{15}N$ lower than expected for an upper trophic level
471 consumer (McMahon and McCarthy 2016). Unless this potential bias is accounted for, trophic
472 position may be substantially underestimated (Dale et al. 2011; Nielsen et al. 2015).
473 Among source amino acids in control sharks, we found a continuum of $\Delta^{15}N$ values,
474 similar to previous studies (McMahon and McCarthy 2016). Only the discrimination factor for
475 phenylalanine met our expectation of not differing from zero, emphasizing its role as a true

476 source amino acid that tracks the $\delta^{15}N$ value of producers at the base of the food web. The other
477 source amino acids (lysine, tyrosine) had discrimination factors that differed from zero and
478 overlapped with at least one trophic amino acid. Glycine also had a positive discrimination
479 factor, and for control sharks serine exhibited a surprisingly negative discrimination factor; this
480 reinforces the recent conclusion that these two amino acids should not be classified as "source"
481 because of their highly-variable discrimination factors in different systems (McMahon and
482 McCarthy 2016). In combination, the patterns in our data suggest that studies of elasmobranchs
483 should consider phenylalanine as the most reliable source amino acid, although this conclusion
484 should be tested in other elasmobranch species.
485
486 *Conclusion.* Elasmobranchs have important ecological roles and can structure marine
487 communities (Young et al. 2015; Bird et al. 2018). Stable isotope analysis can be a powerful tool
488 for assessing these roles (Hussey et al. 2011; Shiffman et al. 2012) but an understanding of how
489 the unique physiology of elasmobranchs influences tissue isotopic patterns is needed to better
490 interpret data collected from wild populations. Overall, we observed higher than expected $\Delta^{13}C$
491 values for essential amino acids (possibly because of microbial contributions), lower than
492 expected $\Delta^{15}N$ values for trophic amino acids (likely because of urea recycling), and evidence
493 that turnover in muscle is slow enough such that shark diet likely cannot be resolved at sub-
494 annual time scales, an issue that can be exacerbated by switching among diet items which differ
495 substantially in $\delta^{13}C$ or $\delta^{15}N$ values. Future studies of free-ranging elasmobranchs should
496 account for these influences when inferring diet composition, trophic level, and habitat use.
497 When these questions are addressed with amino acid isotope data, researchers can include
498 sensitivity analyses of how their conclusions vary after adjusting discrimination factors based on

499 our results. This will help illustrate the capabilities and limitations of isotope-based approaches
500 in ecology.
501
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References

Ballantyne JS (1997) Jaws: the inside story. The metabolism of elasmobranch fishes. Comp Biochem Physiol B Biochem Mol Biol 118:703–742. doi: 10.1016/S0305-0491(97)00272-1

Bird CS, Veríssimo A, Magozzi S, et al (2018) A global perspective on the trophic geography of sharks. Nat Ecol Evol 2:299–305. doi: 10.1038/s41559-017-0432-z

Bradley CJ, Madigan DJ, Block BA, Popp BN (2014) Amino acid isotope incorporation and enrichment factors in Pacific Bluefin Tuna, *Thunnus orientalis*. PLOS ONE 9:e85818. doi: 10.1371/journal.pone.0085818

Cantalapiedra-Hijar G, Dewhurst RJ, Cheng L, et al (2017) Nitrogen isotopic fractionation as a biomarker for nitrogen use efficiency in ruminants: a meta-analysis. Animal 1–11. doi: 10.1017/S1751731117003391

Carleton SA, Martínez del Rio C (2010) Growth and catabolism in isotopic incorporation: a new formulation and experimental data. Funct Ecol 24:805–812

Chandrashekar K, Deosthale YG (1993) Proximate composition, amino acid, mineral, and trace element content of the edible muscle of 20 Indian fish species. J Food Compos Anal 6:195–200

Chikaraishi Y, Kashiyama Y, Ogawa NO, et al (2007) Metabolic control of nitrogen isotope composition of amino acids in macroalgae and gastropods: implications for aquatic food web studies. Mar Ecol Prog Ser 342:85–90. doi: 10.3354/meps342085

Dale J, Wallsgrove N, Popp B, Holland K (2011) Nursery habitat use and foraging ecology of the brown stingray *Dasyatis lata* determined from stomach contents, bulk and amino acid stable isotopes. Mar Ecol Prog Ser 433:221–236. doi: 10.3354/meps09171

Diniz FM, Martin AM (1997) Optimization of nitrogen recovery in the enzymatic hydrolysis of dogfish (*Squalus acanthias*) protein. Composition of the hydrolysates. Int J Food Sci Nutr 48:191–200. doi: 10.3109/09637489709012592

Downs E, Popp B, Holl C (2014) Nitrogen isotope fractionation and amino acid turnover rates in the Pacific white shrimp *Litopenaeus vannamei*. Mar Ecol Prog Ser 516:239–250. doi: 10.3354/meps11030

Engel MH, Hare PE (1985) Gas-liquid chromatographic separation of amino acids and their derivatives. In: Barrett GC (ed) Chemistry and Biochemistry of the Amino Acids. Springer Netherlands, pp 462–479

Fantle MS, Dittel AI, Schwalm SM, et al (1999) A food web analysis of the juvenile blue crab, *Callinectes sapidus*, using stable isotopes in whole animals and individual amino acids. Oecologia 120:416–426. doi: 10.1007/s004420050874

547 Fauconneau B, Arnal M (1985) In vivo protein synthesis in different tissues and the whole body
548 of rainbow trout (*Salmo gairdnerii* R.). Influence of environmental temperature. Comp
549 Biochem Physiol A 82:179–187. doi: 10.1016/0300-9629(85)90723-6

550 Florin ST, Felicetti LA, Robbins CT (2011) The biological basis for understanding and
551 predicting dietary-induced variation in nitrogen and sulphur isotope ratio discrimination.
552 Funct Ecol 25:519–526

553 Galván DE, Jañez J, Irigoyen AJ (2016) Estimating tissue-specific discrimination factors and
554 turnover rates of stable isotopes of nitrogen and carbon in the smallnose fanskate
555 *Sympterygia bonapartii* (Rajidae): estimating tdf values in *s. bonapartii*. J Fish Biol
556 89:1258–1270. doi: 10.1111/jfb.13024

557 Germain LR, Koch PL, Harvey J, McCarthy MD (2013) Nitrogen isotope fractionation in amino
558 acids from harbor seals: implications for compound-specific trophic position calculations.
559 Mar Ecol Prog Ser 482:265–277. doi: 10.3354/meps10257

560 Givens C, Ransom B, Bano N, Hollibaugh J (2015) Comparison of the gut microbiomes of 12
561 bony fish and 3 shark species. Mar Ecol Prog Ser 518:209–223. doi: 10.3354/meps11034

562 Grimes DJ, Brayton P, Colwell RR, Gruber SH (1985) Vibrios as autochthonous flora of neritic
563 sharks. Syst Appl Microbiol 6:221–226. doi: 10.1016/S0723-2020(85)80056-4

564 Hoen DK, Kim SL, Hussey NE, et al (2014) Amino acid ^{15}N trophic enrichment factors of four
565 large carnivorous fishes. J Exp Mar Biol Ecol 453:76–83. doi:
566 10.1016/j.jembe.2014.01.006

567 Howland MR, Corr LT, Young SMM, et al (2003) Expression of the dietary isotope signal in the
568 compound-specific $\delta^{13}C$ values of pig bone lipids and amino acids. Int J Osteoarchaeol
569 13:54–65. doi: 10.1002/oa.658

570 Hughes KL, Whiteman JP, Newsome SD (2018) The relationship between dietary protein
571 content, body condition, and $\Delta^{15}N$ in a mammalian omnivore. Oecologia 186:357–367.
572 doi: 10.1007/s00442-017-4010-5

573 Hussey NE, Brush J, McCarthy ID, Fisk AT (2010) $\delta^{15}N$ and $\delta^{13}C$ diet–tissue discrimination
574 factors for large sharks under semi-controlled conditions. Comp Biochem Physiol A Mol
575 Integr Physiol 155:445–453. doi: 10.1016/j.cbpa.2009.09.023

576 Hussey NE, Dudley SFJ, McCarthy ID, et al (2011) Stable isotope profiles of large marine
577 predators: viable indicators of trophic position, diet, and movement in sharks? Can J Fish
578 Aquat Sci 68:2029–2045. doi: 10.1139/f2011-115

579 Jhaveri P, Papastamatiou YP, German DP (2015) Digestive enzyme activities in the guts of
580 bonnethead sharks (*Sphyrna tiburo*) provide insight into their digestive strategy and
581 evidence for microbial digestion in their hindguts. Comp Biochem Physiol A Mol Integr
582 Physiol 189:76–83. doi: 10.1016/j.cbpa.2015.07.013

583 Jim S, Jones V, Ambrose SH, Evershed RP (2006) Quantifying dietary macronutrient sources of
584 carbon for bone collagen biosynthesis using natural abundance stable carbon isotope
585 analysis. Br J Nutr 95:1055–1062. doi: 10.1079/BJN20051685

586 Kelly LJ, Martínez del Rio C (2010) The fate of carbon in growing fish: an experimental study of
587 isotopic routing. Physiol Biochem Zool 83:473–480

588 Kim SL, Casper DR, Galván-Magaña F, et al (2012a) Carbon and nitrogen discrimination factors
589 for elasmobranch soft tissues based on a long-term controlled feeding study. Environ Biol
590 Fishes 95:37–52. doi: 10.1007/s10641-011-9919-7

591 Kim SL, Koch PL (2012) Methods to collect, preserve, and prepare elasmobranch tissues for
592 stable isotope analysis. Environ Biol Fishes 95:53–63. doi: 10.1007/s10641-011-9860-9

593 Kim SL, Martínez del Rio C, Casper D, Koch PL (2012b) Isotopic incorporation rates for shark
594 tissues from a long-term captive feeding study. J Exp Biol 215:2495–2500. doi:
595 10.1242/jeb.070656

596 Knight IT, Grimes DJ, Colwell RR (1988) Bacterial hydrolysis of urea in the tissues of
597 carcharhinid sharks. Can J Fish Aquat Sci 45:357–360

598 Lee TN, Buck CL, Barnes BM, O'Brien DM (2012) A test of alternative models for increased
599 tissue nitrogen isotope ratios during fasting in hibernating arctic ground squirrels. J Exp
600 Biol 215:3354–3361. doi: 10.1242/jeb.068528

601 Lorrain A, Graham BS, Popp BN, et al (2015) Nitrogen isotopic baselines and implications for
602 estimating foraging habitat and trophic position of yellowfin tuna in the Indian and
603 Pacific Oceans. Deep Sea Res Part II Top Stud Oceanogr 113:188–198. doi:
604 10.1016/j.dsr2.2014.02.003

605 Malpica-Cruz L, Herzka SZ, Sosa-Nishizaki O, et al (2012) Tissue-specific isotope trophic
606 discrimination factors and turnover rates in a marine elasmobranch: empirical and
607 modeling results. Can J Fish Aquat Sci 69:551–564. doi: 10.1139/f2011-172

608 Martínez del Rio C, Carleton SA (2012) How fast and how faithful: the dynamics of isotopic
609 incorporation into animal tissues. J Mammal 93:353–359

610 Martínez del Rio CM, Wolf N, Carleton SA, Gannes LZ (2009) Isotopic ecology ten years after a
611 call for more laboratory experiments. Biol Rev 84:91–111

612 McMahon KW, Fogel ML, Elsdon TS, Thorrold SR (2010) Carbon isotope fractionation of
613 amino acids in fish muscle reflects biosynthesis and isotopic routing from dietary protein.
614 J Anim Ecol 79:1132–1141. doi: 10.1111/j.1365-2656.2010.01722.x

615 McMahon KW, McCarthy MD (2016) Embracing variability in amino acid $\delta^{15}N$ fractionation:
616 mechanisms, implications, and applications for trophic ecology. Ecosphere 7:e01511.
617 doi: 10.1002/ecs2.1511

618 McMahon KW, Thorrold SR, Elsdon TS, McCarthy MD (2015) Trophic discrimination of
619 nitrogen stable isotopes in amino acids varies with diet quality in a marine fish: Trophic
620 discrimination of amino acids. Limnol Oceanogr 60:1076–1087. doi: 10.1002/lno.10081

621 Newsome SD, Fogel ML, Kelly L, Martínez del Rio C (2011) Contributions of direct
622 incorporation from diet and microbial amino acids to protein synthesis in Nile tilapia.
623 Funct Ecol 25:1051–1062. doi: 10.1111/j.1365-2435.2011.01866.x

624 Newsome SD, Wolf N, Peters J, Fogel ML (2014) Amino acid $\delta^{13}C$ analysis shows flexibility in
625 the routing of dietary protein and lipids to the tissue of an omnivore. Integr Comp Biol
626 54:890–902. doi: 10.1093/icb/icu106

627 Nielsen JM, Popp BN, Winder M (2015) Meta-analysis of amino acid stable nitrogen isotope
628 ratios for estimating trophic position in marine organisms. Oecologia 178:631–642. doi:
629 10.1007/s00442-015-3305-7

630 O'Connell TC (2017) 'Trophic' and 'source' amino acids in trophic estimation: a likely
631 metabolic explanation. Oecologia 184:317–326. doi: 10.1007/s00442-017-3881-9

632 Onodenalore AC, Shahidi F (1996) Protein dispersions and hydrolysates from sharks (*Isurus*
633 *oxyrinchus*). J Aquat Food Prod Technol 5:43–59

634 Pauly D (1980) On the interrelationships between natural mortality, growth parameters, and
635 mean environmental temperature in 175 fish stocks. ICES J Mar Sci 39:175–192. doi:
636 10.1093/icesjms/39.2.175

637 Post DM, Layman CA, Arrington DA, et al (2007) Getting to the fat of the matter: models,
638 methods and assumptions for dealing with lipids in stable isotope analyses. Oecologia
639 152:179–189

640 Robbins CT, Felicetti LA, Sponheimer M (2005) The effect of dietary protein quality on nitrogen
641 isotope discrimination in mammals and birds. Oecologia 144:534–540

642 Shiffman DS, Gallagher AJ, Boyle MD, et al (2012) Stable isotope analysis as a tool for
643 elasmobranch conservation research: a primer for non-specialists. Mar Freshw Res
644 63:635. doi: 10.1071/MF11235

645 Stevens CE, Hume ID (1998) Contributions of microbes in vertebrate gastrointestinal tract to
646 production and conservation of nutrients. Physiol Rev 78:393–427

647 USDA National Nutrient Database for Standard Reference Legacy Release.
648 https://ndb.nal.usda.gov/ndb/foods/show/303613?fgcd=&manu=&lfacet=&format=&cou
649 nt=&max=25&offset=&sort=default&order=asc&qlookup=tilapia&ds=&qt=&qp=&qa=
650 &qn=&q=&ing=. Accessed 18 Apr 2018

651 Vander Zanden MJ, Clayton MK, Moody EK, et al (2015) Stable isotope turnover and half-life
652 in animal tissues: a literature synthesis. PLoS ONE 10:e0116182. doi:
653 10.1371/journal.pone.0116182

Vokhshoori NL, McCarthy MD (2014) Compound-specific $\delta^{15}N$ amino acid measurements in littoral mussels in the California Upwelling ecosystem: A new approach to generating baseline $\delta^{15}N$ isoscapes for coastal ecosystems. PLOS ONE 9:e98087. doi: 10.1371/journal.pone.0098087

Wood CM, Giacomin M (2016) Feeding through your gills and turning a toxicant into a resource: how the dogfish shark scavenges ammonia from its environment. J Exp Biol 219:3218–3226. doi: 10.1242/jeb.145268

Wu G (1998) Intestinal mucosal amino acid catabolism. J Nutr 128:4p

Young JW, Hunt BPV, Cook TR, et al (2015) The trophodynamics of marine top predators: Current knowledge, recent advances and challenges. Deep Sea Res Part II Top Stud Oceanogr 113:170–187. doi: 10.1016/j.dsr2.2014.05.015

Zeichner SS, Colman AS, Koch PL, et al (2017) Discrimination factors and incorporation rates for organic matrix in shark teeth based on a captive feeding study. Physiol Biochem Zool 90:257–272. doi: 10.1086/689192

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).

Classification	Amino Acid	Shark	Squid	Tilapia
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

677 Table 2. Test statistics for linear regression models in which amino acid imbalance between
678 leopard sharks and their diet (measured as the difference in mole percent) was the predictor of
679 stable isotope discrimination factors ($\Delta^{13}C$ and $\Delta^{15}N$) between leopard sharks and their diet. Two
680 groups of sharks were evaluated: control (fed a constant diet of squid for 1250 days) and
681 experimental (diet switched to tilapia on day 565). One model exhibited a significant relationship
682 as indicated by an asterisk. However, after removal of a single data point (serine) on the basis of
683 a high Cook's Distance score, the relationship was not significant.
684

Predictor	**Response**	**Group**	**Amino acids**	**N**	***P***	**F**	**R^2**
Amino acid imbalance	$\Delta^{13}C$	Control	All	12	0.95	0.00	<0.01
		Control	Non-essential	6	0.66	0.22	<0.01
	$\Delta^{15}N$	Control	All	12	0.52	0.44	<0.01
		Control	Trophic	6	0.93	0.01	<0.01
	$\Delta^{13}C$	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
	$\Delta^{15}N$	Experimental	All	12	0.66	0.21	<0.01
		Experimental	Trophic	6	0.96	0.00	<0.01

685
686

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

691

692 Figure 1. Conceptual figure illustrating the effect of incomplete turnover on the appearance of
693 carbon isotope discrimination factors ($\Delta^{13}C_{consumer - diet}$). The concepts apply to other isotopes as
694 well. a–c) Each panel represents the $\delta^{13}C$ values of an amino acid (X, Y, or Z) measured in the
695 tissue of a consumer (black line) that switched from an old diet to a new diet (dashed gray lines)
696 on day 0. Each amino acid had the same turnover rate and if the consumer was in a steady state
697 with its diet a $\Delta^{13}C$ value of 2‰. The y-axis is identical across panels. The differences in $\delta^{13}C$
698 values between the old diet and new diet cause $\Delta^{13}C$ to differ between days 100 (Δ_{100}) and 500
699 (Δ_{500}). d) On day 100, amino acid X already had a $\Delta^{13}C$ value that was close to 2‰ because the
700 old diet and new diet were similar in $\delta^{13}C$ values. However, for amino acid Z, $\Delta^{13}C$ was much
701 larger than 2‰ because the old diet and new diet differed substantially in $\delta^{13}C$ values. e) On day
702 500, when turnover was nearly complete, each amino acid had a $\Delta^{13}C$ value of about 2‰.
703
704 Figure 2. Carbon isotope values of individual amino acids in leopard shark muscle and in diet
705 items of the sharks. Box plots indicate median (solid line) and 25th, 10th, and 5th percentiles. a)
706 $\delta^{13}C$ values of control sharks (fed squid for 1250 days; N = 3), experimental sharks (switched to
707 a diet of tilapia on day 565; N = 6), and their respective diet items (squid, N = 3; tilapia, N = 2).
708 b) Discrimination factors ($\Delta^{13}C$) for control and experimental sharks. Asterisks above boxes
709 indicate *P* values for a one-tailed t-test of whether discrimination factors differed from zero (test
710 statistics listed in Table S2). Brackets and asterisks below boxes indicate *P* values for a two-
711 tailed t-test of whether control and experimental sharks differed (test statistics listed in Table
712 S3).
713

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) $\delta^{13}C$ and b) $\delta^{15}N$. 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 25^{th}, 10^{th}, and 5^{th} percentiles. a) $\delta^{15}N$ 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., 10^{th} percentile for the experimental sharks). b) Discrimination factors ($\Delta^{15}N$) 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).

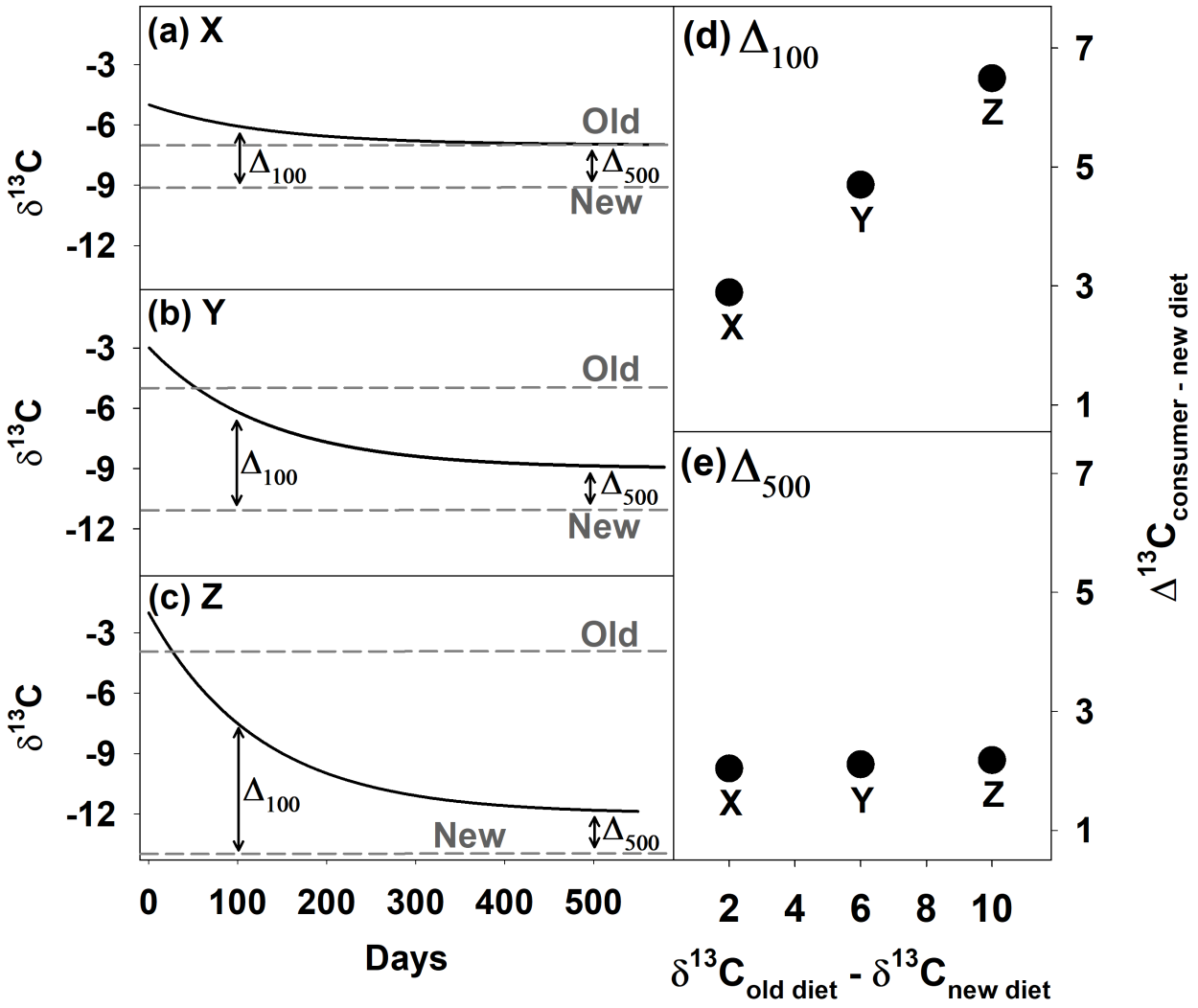
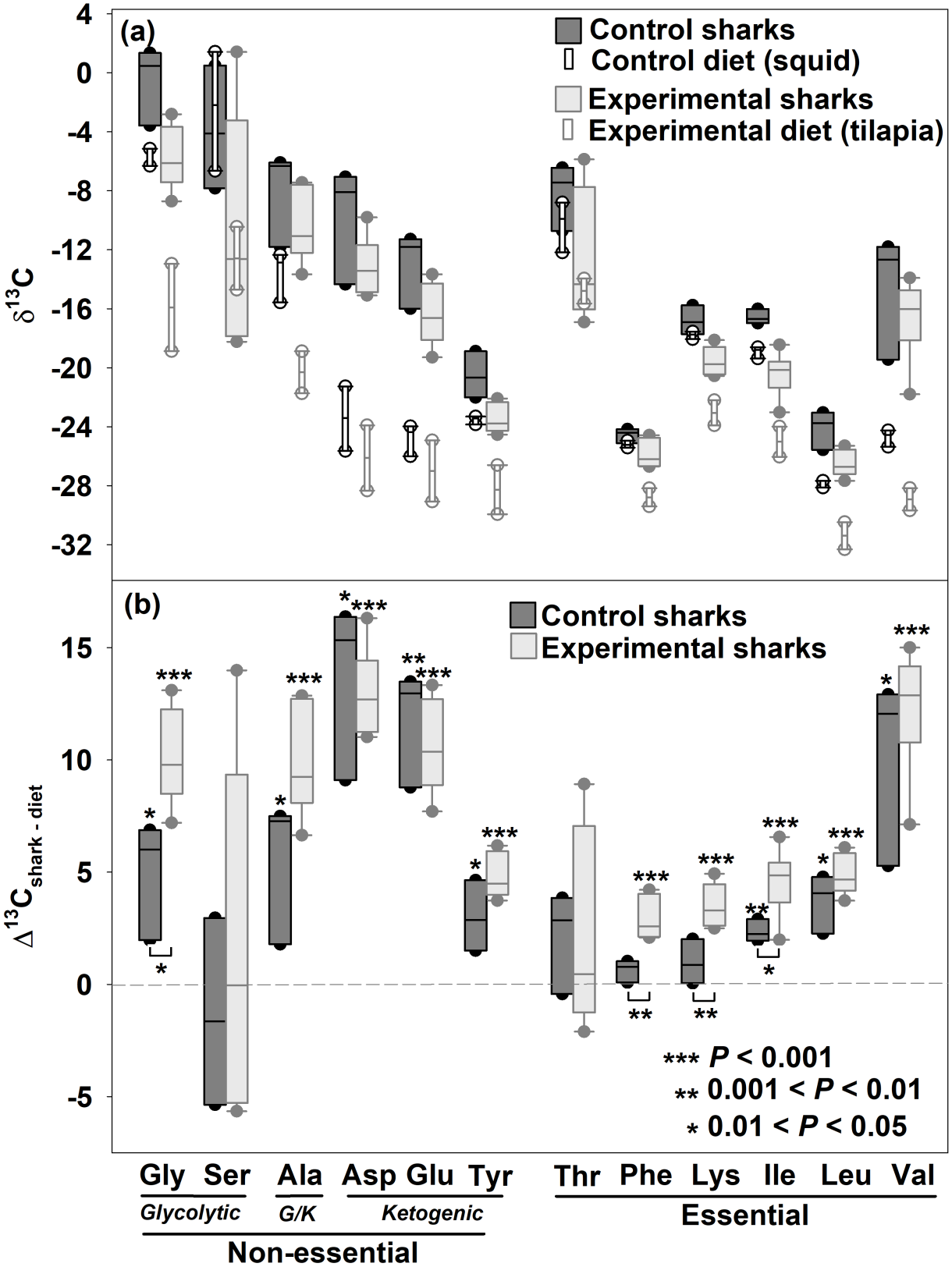
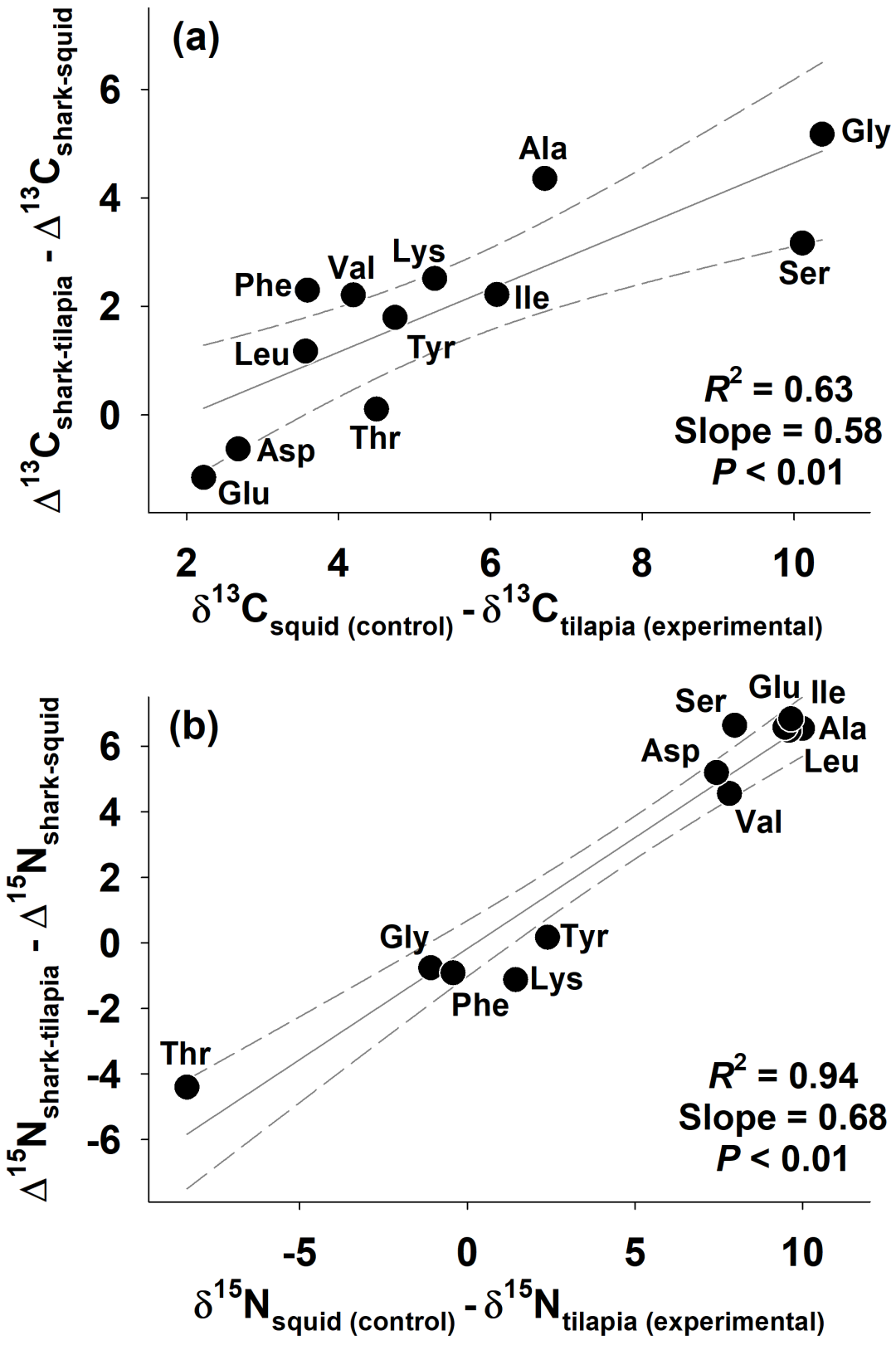

(a) X
(b) Y
(c) Z
(d) Δ100
(e) Δ500
Old
New
Δ100
Δ500
δ13C
-3
-6
-9
-12
0
100
200
300
400
500
Days
X
Y
Z
2
4
6
8
10
δ13Cold diet - δ13Cnew diet
7
5
3
1
Δ13Cconsumer - new diet

Figure 1.



746

747 Figure 2.



748

749 Figure 3.

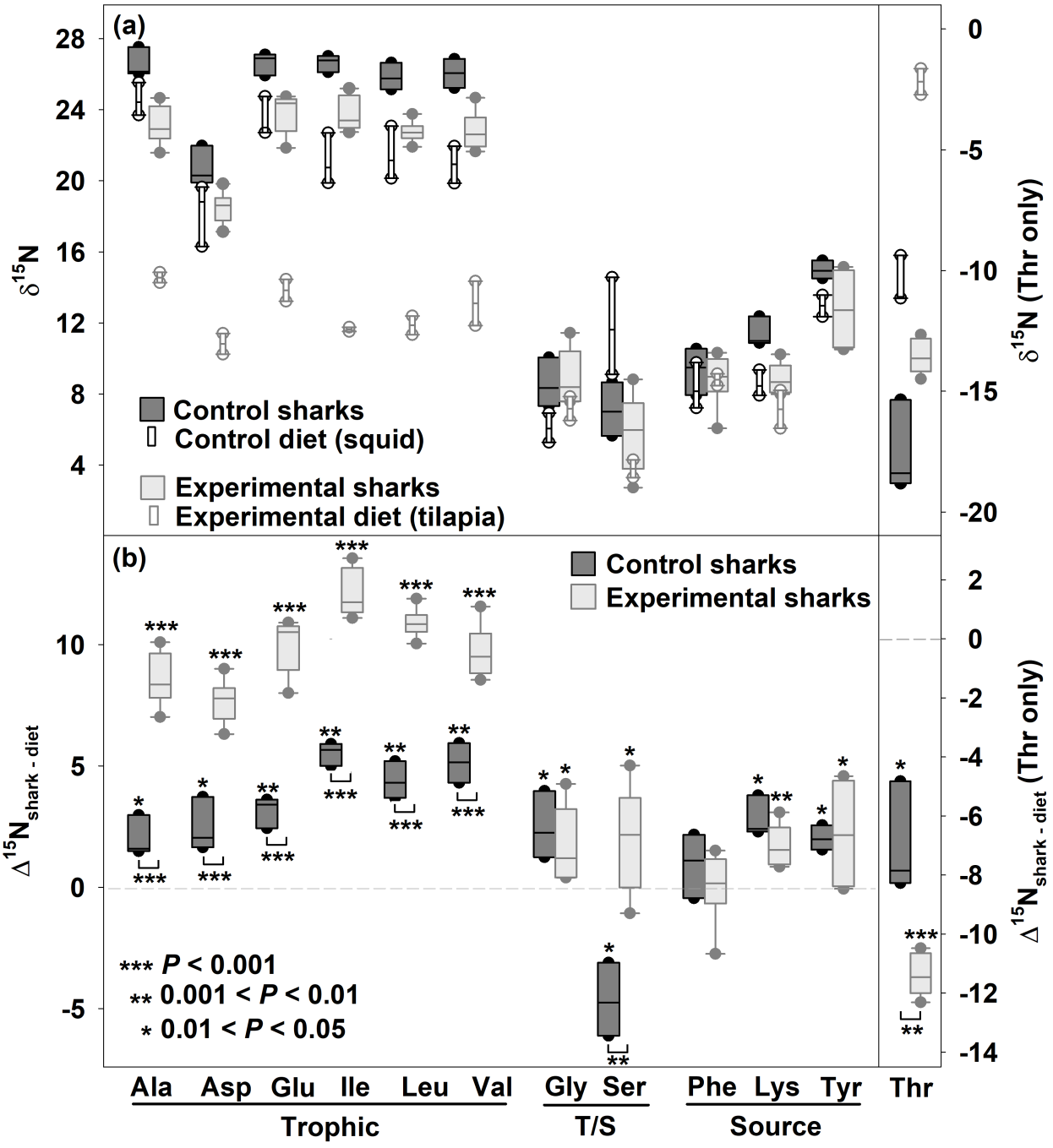

(a)
(b)
δ15N
δ15N (Thr only)
Δ15Nshark - diet
Δ15Nshark - diet (Thr only)
Control sharks
Control diet (squid)
Experimental sharks
Experimental diet (tilapia)
Control sharks
Experimental sharks
*** P < 0.001
** 0.001 < P < 0.01
* 0.01 < P < 0.05
Ala Asp Glu Ile Leu Val
Gly Ser
Phe Lys Tyr
Thr
Trophic
T/S
Source

Figure 4.

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

Supplementary Materials

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

Shark ID	Experimental day	$\delta^{13}C$	$\delta^{15}N$
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	$\delta^{13}C$	$\delta^{15}N$
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	$\delta^{13}C$	$\delta^{15}N$
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	$\delta^{13}C$	$\delta^{15}N$
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	$\delta^{13}C$	$\delta^{15}N$
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	$\delta^{13}C$	$\delta^{15}N$
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	$\delta^{13}C$	$\delta^{15}N$
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	$\delta^{13}C$	$\delta^{15}N$
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	$\delta^{13}C$	$\delta^{15}N$
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	*P*	t	DF
$\Delta^{13}C$	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
	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
$\Delta^{15}N$	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	*P*	t	DF
$\Delta^{15}N$	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.

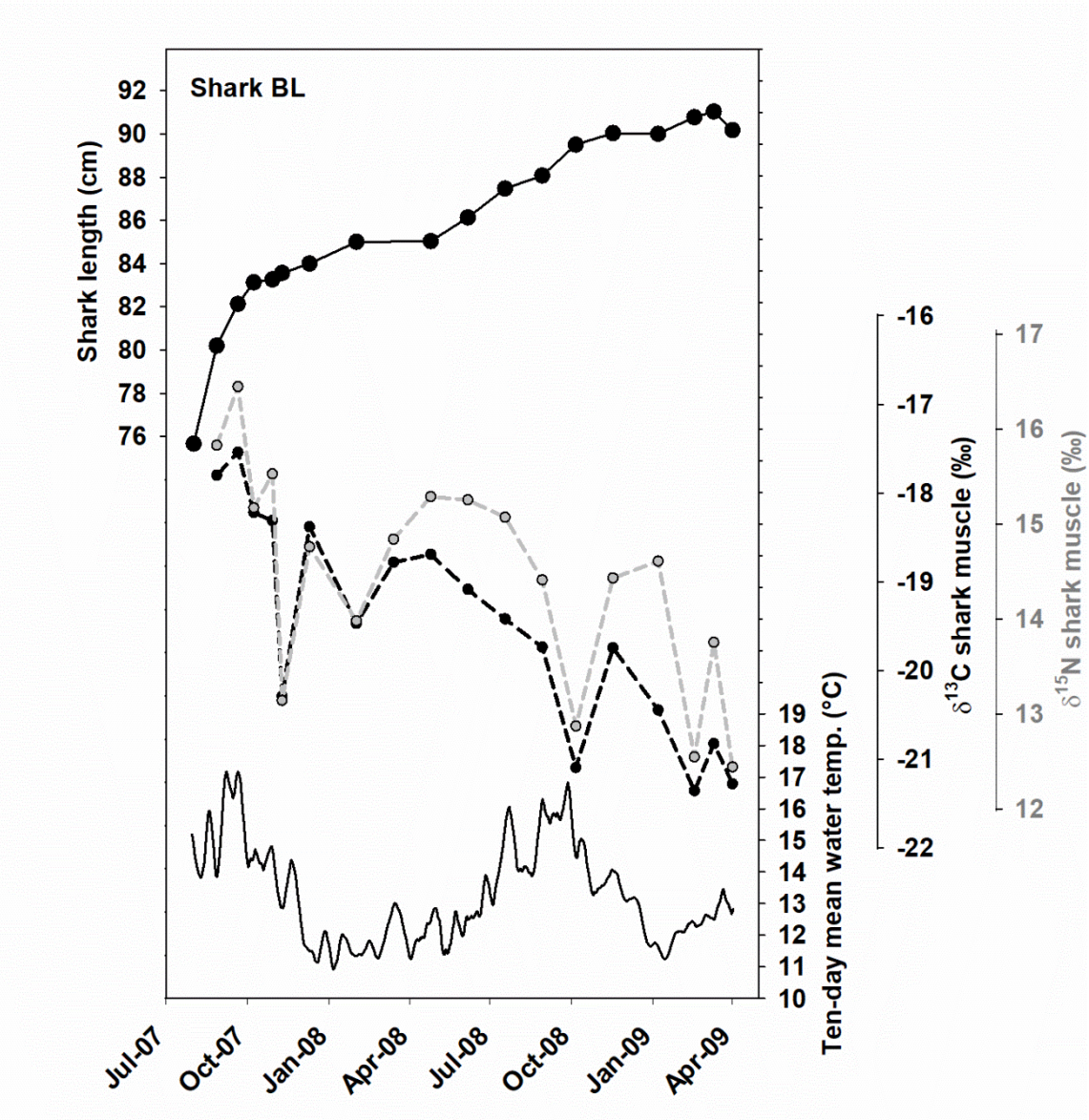
	Amino acid	***P***	**t**	**DF**
$\Delta^{13}C$	Alanine	0.056	-2.289	7
	Glycine	0.015	-3.190	7
	Threonine	0.971	-0.038	7
	Serine	0.535	-0.652	7
	Valine	0.381[a]	11.000[a]	NA[a]
	Leucine	0.149	-1.619	7
	Isoleucine	0.045	-2.431	7
	Aspartic acid	0.753	0.327	7
	Glutamic acid	0.488	0.731	7
	Phenylalanine	0.003	-3.844	7
	Tyrosine	0.069	-2.144	7
	Lysine	0.008	-3.691	7
$\Delta^{15}N$	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

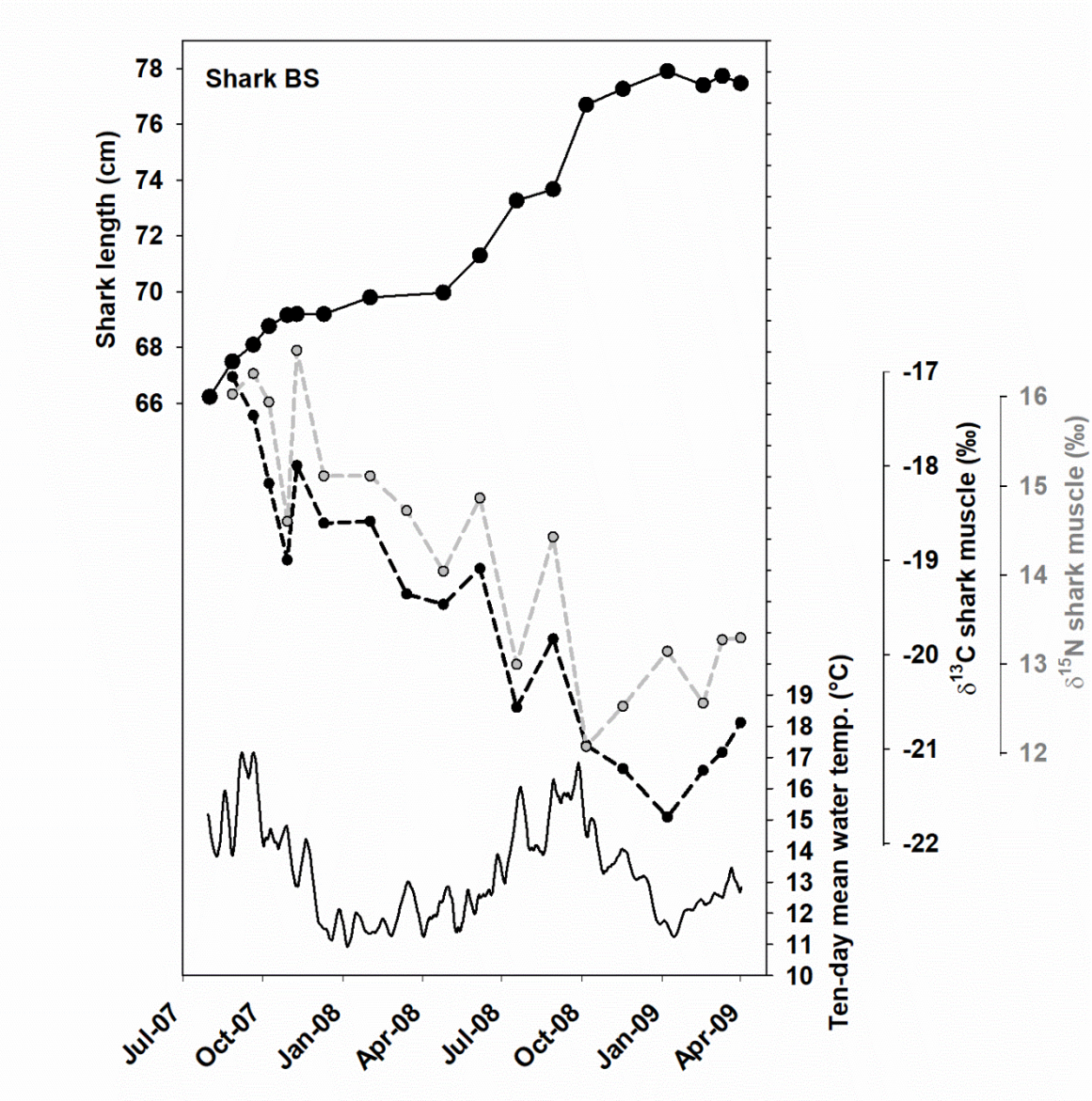
[a]Statistics from Mann-Whitney rank sum test because data failed Shapiro-Wilk test of normality.

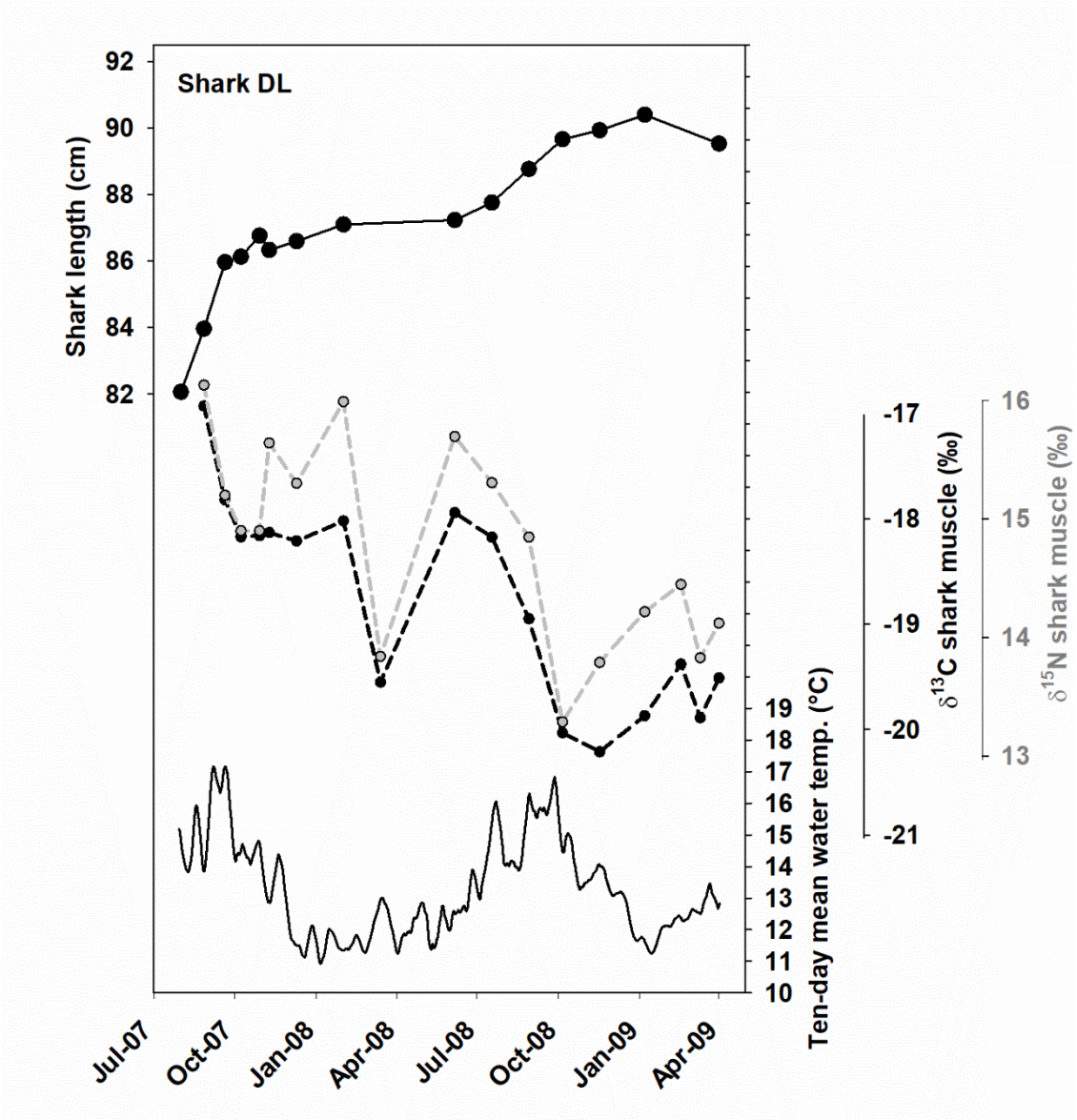
Figure S1. Data from six leopard sharks (individuals identified by a two-letter code) used in this captive feeding study. Measurements include body length (solid black line and circles) and stable isotope values of bulk muscle tissue ($\delta^{13}C$ values are black dashed line and circles, $\delta^{15}N$ 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).

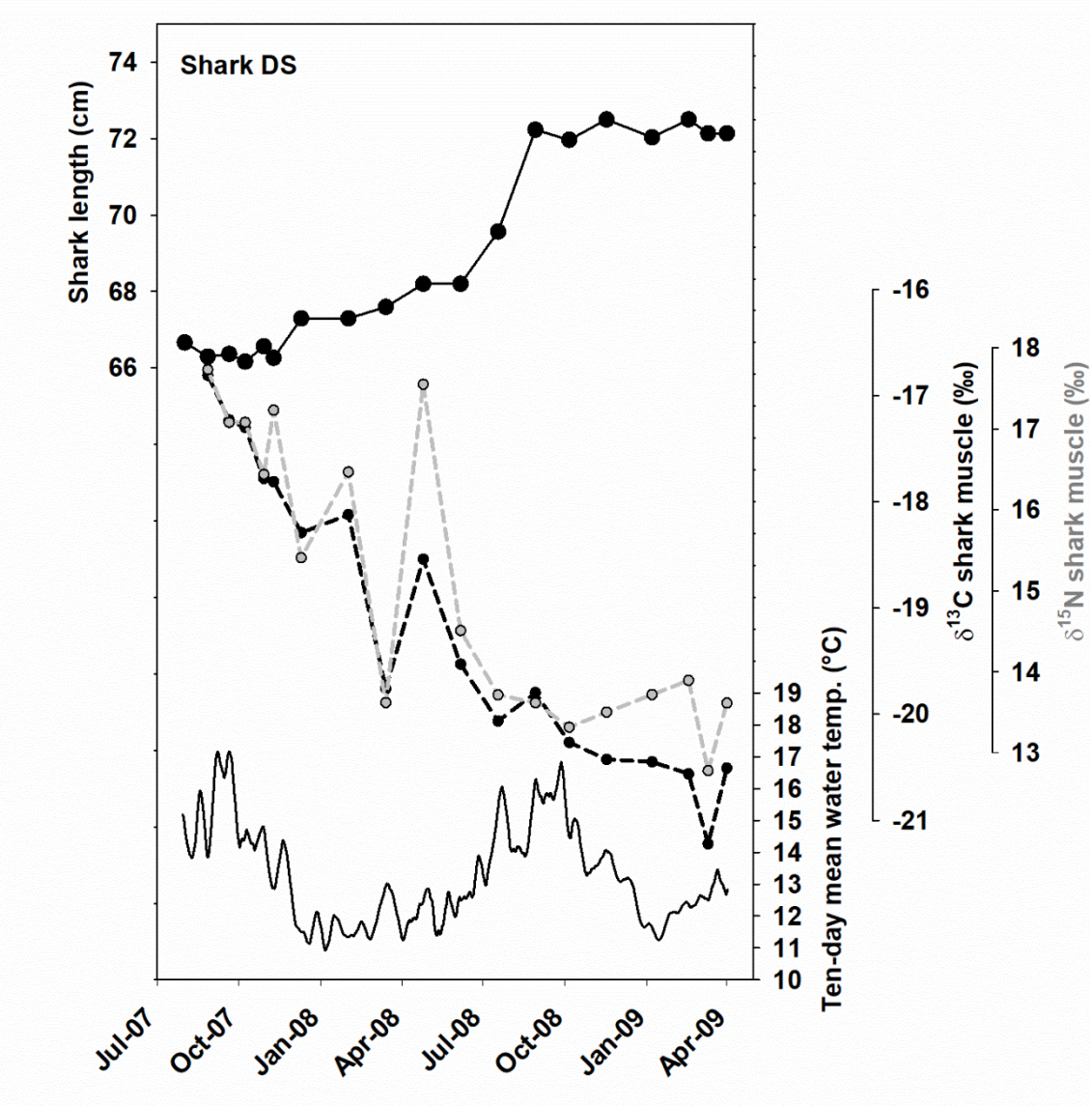
Reference

Kim SL, Martínez del Rio 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.




Shark BS
Shark length (cm)
78
76
74
72
70
68
66
Ten-day mean water temp. (°C)
19
18
17
16
15
14
13
12
11
10
δ13C shark muscle (‰)
-17
-18
-19
-20
-21
-22
δ15N shark muscle (‰)
16
15
14
13
12
Jul-07
Oct-07
Jan-08
Apr-08
Jul-08
Oct-08
Jan-09
Apr-09


Shark DL
Shark length (cm)
92
90
88
86
84
82
Ten-day mean water temp. (°C)
19
18
17
16
15
14
13
12
11
10
δ13C shark muscle (‰)
-17
-18
-19
-20
-21
δ15N shark muscle (‰)
16
15
14
13
Jul-07
Oct-07
Jan-08
Apr-08
Jul-08
Oct-08
Jan-09
Apr-09


Shark DS
Shark length (cm)
74
72
70
68
66
Ten-day mean water temp. (°C)
19
18
17
16
15
14
13
12
11
10
δ13C shark muscle (‰)
-16
-17
-18
-19
-20
-21
δ15N shark muscle (‰)
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15
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13
Jul-07
Oct-07
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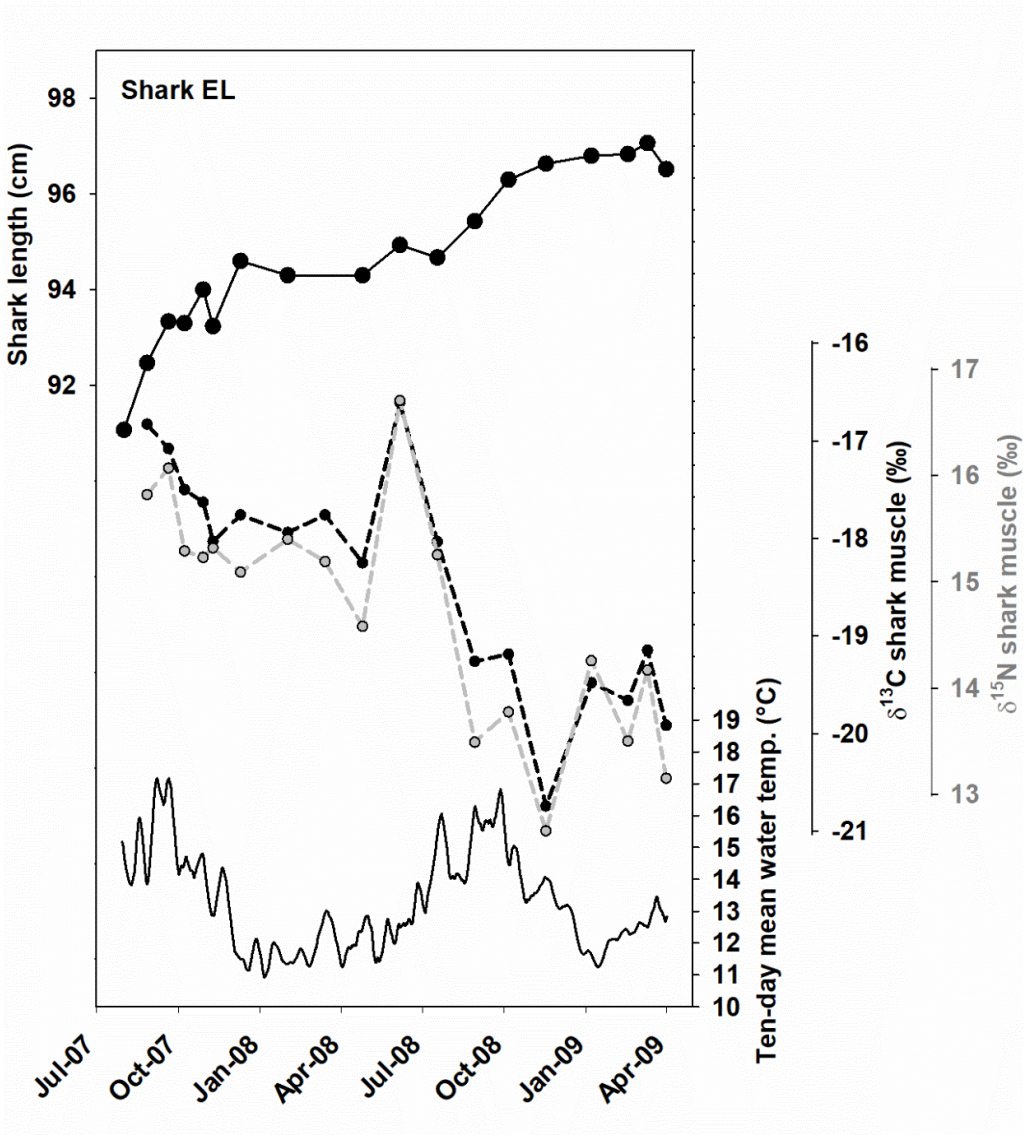
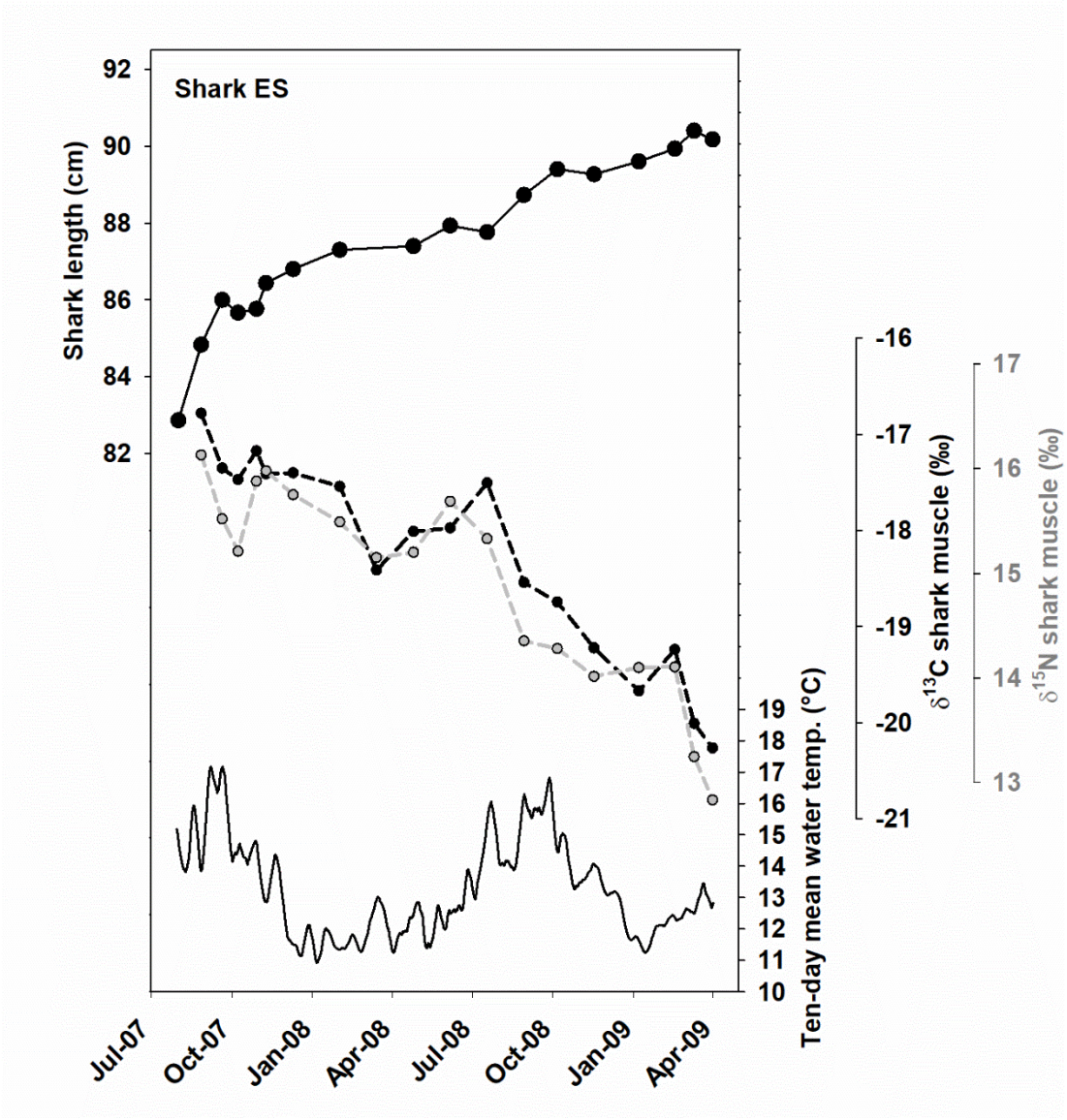
Apr-09


Shark EL
Shark length (cm)
98
96
94
92
Jul-07
Oct-07
Jan-08
Apr-08
Jul-08
Oct-08
Jan-09
Apr-09
Ten-day mean water temp. (°C)
19
18
17
16
15
14
13
12
11
10
δ13C shark muscle (‰)
-16
-17
-18
-19
-20
-21
δ15N shark muscle (‰)
17
16
15
14
13


Shark ES
Shark length (cm)
92
90
88
86
84
82
Ten-day mean water temp. (°C)
19
18
17
16
15
14
13
12
11
10
δ13C shark muscle (‰)
-16
-17
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-21
δ15N shark muscle (‰)
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Figure S2. Consumer-diet discrimination factors for stable nitrogen isotopes ($\Delta^{15}N$) 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.