Trophic Enrichment Factors of Stables Isotope and Fatty Acid Biomarkers in the Blue Crab, *Callinectes sapidus*

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**Abstract**

Stable isotope and fatty acid biomarkers provide new ways to understand predator-prey interactions. In particular, quantitative inference about the strength of feeding links can be achieved with biomarker mixing models, whose accurate application requires knowledge of trophic enrichment factors (TEF) accounting for metabolism, deposition, or synthesis (also known as a “discrimination factors” or “calibration coefficients” for stable isotopes and fatty acids, respectively). Nevertheless, many applications of mixing models do not use TEFs developed for the particular predator or conditions of interest (e.g., life stage, diet quality). We conducted a 12-week laboratory experiment to quantify stable isotope and fatty acid TEFs for the ecologically and economically important blue crab, *Callinectes sapidus.* We calculated TEFs for δ13C and δ15N stable isotopes and 37 unique fatty acids in the claw muscle and hepatopancreas of crabs fed clams or black sea bass. For both tissues and diets, δ13C TEFs were near zero, whereas δ15N TEFs were influenced by diet. The multivariate fatty acid profile diverged from initial values towards the fatty acid profile of the diet over the course of the experiment. In general, the pattern of fatty acid TEFs was similar across diets and more influenced by tissue type. Some fatty acids with exceptionally high TEFs include 18:2n-6-cis, 21:0, and 23:0; low TEF fatty acids include 20:2n-11,14-cis and 20:4n-3. Although this is among the only studies to quantify both stable isotope and fatty acid TEFs in a marine crab, further controlled feeding studies are needed to determine the influence of a wide range of covariates on these important parameters.

**Introduction**

Despite being one of the earliest and most central concepts in ecology, interest in food webs continues to rise [1-2]. The modern increase in research activity on food webs has likely been enabled by an expanding toolbox for trophic ecologists. While most early food webs were assembled via analysis of gut contents, examination of scat or fecal pellets, direct observation of consumer-resource interactions, or other types of natural history knowledge [e.g. 3-5], several new techniques have developed in recent decades. In particular, stable isotopes and fatty acids can serve as trophic biomarkers that have many advantages for elucidating predator-prey interactions compared to traditional techniques [reviewed by 6-7]. The basic premise behind the use of trophic biomarkers is that the concentration (or value) of a particular biomarker in a consumer will reflect the values of that biomarker in its resources, with some modification [8]. In general, these trophic biomarkers provide longer temporal integration of a consumer’s diet and circumvent certain data biases of traditional methods (e.g. unequal digestibility, coarse resolution of diet items, inability to directly observe feeding in highly mobile organisms) [5,9].

In many cases, a mixing model is used to make a quantitative estimate of a consumer’s diet using trophic biomarker values in the consumer and its potential resources. Generally, mixing models use either stable isotopes or fatty acids [e.g. 10-12, but see 13 and Landsman et al. 2018, which makes inference with both biomarkers]. In most mixing models, a trophic enrichment factor (TEF) is applied to account for metabolism, deposition, synthesis, or other factors that might cause the value of the biomarker to differ between the consumer and its resource. For example, δ15N values are typically enriched in a consumer relative to its prey due to fractionation of 15N and 14N during excretion of nitrogenous wastes and deposition of nitrogen in tissue (where δ13C or δ15N = [(Rsample / Rstandard)] and R is the isotopic ratio 13C/12C or 15N/14N of the sample and a standard) [14]. For fatty acids, the proportion of a fatty acid in a consumer may not match its proportion in its resources due to metabolism or de-novo synthesis [10]. Therefore, some fatty acids can occur in higher or lower proportions in a predator, relative to its prey, and mixing model inference is often restricted to those fatty acids that are expected to be exclusively dietary in origin [e.g. 15].

We use the term *trophic enrichment factor* (TEF) to refer to resource-to-consumer modification of for both stable isotopes and fatty acids, although specific terms may be used for each biomarker (e.g. *discrimination* or *enrichment factor* for stable isotopes or *calibration* or *conversion coefficient* for fatty acids). In both cases, TEFs are most often determined through laboratory feeding studies where a consumer is given a controlled diet for an extended period of time, until the biomarker value in the consumer has equilibrated. Then, for each fatty acid (FA) the TEF can be calculated as: % FA consumer / % FA resource [10]. TEFs for each stable isotope can be calculated as: δX consumer -δX resource, where X represents C, N or another element [16].

Although many factors are known to modify TEFs [e.g. diet quality, consumer species, age, tissue, or physiological status; 7,16-17], most applications of mixing models use literature-derived values, rather than making estimates for the particular consumer of interest [7]. For example, many studies that attempt to estimate trophic level from δ15N assume a constant enrichment per trophic level (e.g. 3.4 ‰) although considerable variation for this value exists [16]. The use of incorrect TEFs is not without consequences. Inferences made from mixing models are highly-sensitive to the TEFs used for both stable isotopes and fatty acids [e.g. 18-20]. As a result, there have been repeated calls for more laboratory experiments to determine TEFs for a greater number of consumers and to gain a better understanding of the factors that affect deposition, metabolism, and synthesis of biomarkers [20-22, White et al. 2019]. Finally, despite the complementarity of stable isotope and fatty acid biomarkers and new models that can utilize both types of information [13] few experiments quantifying TEFs have measured both simultaneously [but see 23].

This study quantifies TEFs for both stable isotopes and fatty acids in the ecologically and economically important blue crab, *Callinectes sapidus* (Brachyura, Portunidae). The blue crab occurs in estuarine, reef, and salt marsh habitats along the WesternAtlantic Coast from New York to Argentina, as well as introduced populations elsewhere [24]. This species typically occupies an intermediate trophic level, with diet-shifts throughout its life, and is topologically important in many food webs because of numerous interactions as both a predator and prey [25]. Blue crabs are also economically important with commercial and recreational fisheries throughout its range [26]. Previous studies have found considerable variation in TEFs for δ13C and δ15N (i.e., discrimination factors) in juvenile *C. sapidus*, depending mostly on the protein content of the diet and the growth rate of the crab [27-28]. To our knowledge there have been no studies that have estimated TEFs for fatty acids in *C. sapidus,* although some studies have measured the fatty acid composition of various blue crab tissues; especially studies with an interest in the nutritional value of blue crabs for human consumption [e.g. 29-31].

In this study, we estimate the trophic enrichment factors for C and N stable isotopes and fatty acids for *C. sapidus*. To understand the effect of diet on TEFs, we measured these biomarkers in crabs fed either hard clam (*Mercenaria mercenaria*) or black sea bass (*Centropristis striata*) for 12 weeks in the laboratory. We measured these biomarkers in both the hepatopancreas and claw muscle of crabs to understand whether TEFs differ according to tissue type. In particular, these two tissues may have different turnover rates. In endotherms such as mammals and birds, more metabolically active tissues (i.e., liver) frequently have faster turnover than muscle [32-33]. On the other hand, evidence for tissue-specific turnover rates in ectotherms such as fish is mixed [34-35].

**Methods**

**Crab collection**

Blue crabs were collected by seining and crab traps in the vicinity of the Rutgers Marine Field Station (Tuckerton, New Jersey; 39.509, -74.324) in July 2015. Both male and female crabs (n = 87) were used in the experiment and had an average initial carapace width of 62.03 mm (min = 27.39 mm, max = 106.43 mm) and an average initial damp mass of 26.14 g (min = 1.38 g, max = 84.4 g). An additional 22 crabs were collected to provide initial stable isotope and fatty acid values.

**Diet items**

Crabs were fed either hard clams (*Mercenaria mercenaria*) or black sea bass (*Centropristis striata*) daily. Both diet items came from the area where the crabs were collected. Freshly caught and shucked clams caught in Barnegat Bay, New Jersey were purchased from a local bait shop. Fish were collected between 2011 and 2013 from wrecks and reefs within approximately 30 km of Little Egg Inlet (39.499129, -74.305078) and stored at -20 °C. Although black sea bass may not be a likely component of blue crab diet in the wild, these diet items were expected to be nutritionally distinct and were chosen to determine the effect of diet on trophic enrichment factors. The sea bass were filleted and the entire soft body of the clams was used. Each diet item was minced and homogenized and allocated into ice cube trays (each cube was approximately 2.2 mL and 0.43 g dry mass for both diets) and frozen at -80 °C until use in the experiment.

**Experimental conditions**

To avoid cannibalism, crabs were housed individually in 1.75 L containers (approximately 10 x 19 x 11.5 cm). Each container was equipped with a bubbling air stone to maintain dissolved oxygen levels, and containers were cleaned and the water was replaced every day after feeding. Seawater was maintained at a salinity of 32 and an average temperature of 23 °C (range: 21 – 25 °C). Lights were kept on a 14 hour light: 10 hour dark schedule. Molting during the course of the experiment was recorded and any exoskeletons were removed from the containers each day.

**Crab tissue samples**

Crab tissue samples for stable isotope and fatty acid analysis were collected at the start of the experiment (i.e., immediately after collection from the wild, n = 22) and from experimental crabs every two weeks for 12 weeks (n = 3 – 10 crabs per diet treatment at each time point). Crabs were euthanized by freezing at -20 °C, and then moved to -80 °C within three hours. Crab hepatopancreas and claw muscle were removed and analyzed for stable isotopes and fatty acids.

**Stable isotope and fatty acid analysis**

Tissue samples were freeze-dried at -85 °C and lipids were extracted with a modified Folch et al. method [36]. Fatty acids were methylated following the methods of Galloway et al. [37] to yield fatty acid methyl esters (FAME). FAMEs were analyzed on a Shimadzu Gas Chromatograph 2010 with a flame ionization detector and a 60m x 0.25mm ID x 0.25 μm film TR-FAME column from Thermo Scientific. For most samples, the remaining tissue was dried at 60°C, and then ground, weighed (1.0 ± 0.2 mg), and packed into tin capsules for the stable isotope analysis. Analysis of bulk δ13C and δ15N was conducted at the University of California Davis (UCD) Stable Isotope Facility on a PDZ Europa 20-20 isotope ratio mass spectrometer.

Thirty-seven fatty acids were identified by comparison to a standard with a known fatty acid composition and by comparison of retention times to the known peak from nonadecanolic acid (19:0), which is added to each sample to serve as an internal standard but typically does not occur in the samples otherwise. Both -*cis* and *-trans* configurations of fatty acids were identified based on comparisons to standards and confirmed by running duplicate samples on a gas chromatograph – mass spectrometer. The amount of each fatty acid is expressed as a normalized area percentage based on all fatty acids (i.e. according to all peaks, not just the 37 identified fatty acids). The total lipid content (% dry mass) of each sample was calculated based on the peak area of the nonadecanolic acid standard and the dry mass of the sample analyzed.

To determine the effect of lipid extraction on stable isotope values, a subset of samples were split and analyzed only via stable isotope analysis or via stable isotope analysis following lipid extraction. This allowed for the calculation of correction factor to account for lipid extraction, since lipids can be 6 to 8‰ depleted in 13C compared to other molecules [38]. Throughout the text, lipid-extracted stable isotope values are reported, unless otherwise noted.

**Statistical analyses and TEF calculations**

For univariate, continuous response variables (e.g. growth rate, stable isotope concentrations), analysis of variance (ANOVA) was used to test for differences between diets or tissues. We tested all assumptions of ANOVAs (i.e. homogeneity of variances, normally-distributed residuals) and transformed the response variable to meet these assumptions if necessary. To assess differences in the multivariate fatty acid profiles, we used non-metric multidimensional scaling (NMDS) to visualize the data in fewer dimensions and we used analysis of similarities (ANOSIM) to test for differences between groups (e.g. diet items, tissues). Both NMDS and ANOSIM were performed with the R package vegan and used Bray-Curtis distances.

Our experiment allowed us to calculate TEFs for each biomarker (δ13C, δ15N, and each of the 37 fatty acids identified) in both tissues (i.e. claw muscle or hepatopancreas) and on both diets (i.e. clam or fish). TEFs were calculated according to the formulas provided above. To calculate TEFs, we used crabs that were sampled live on weeks 8, 10 and 12 in the case of stable isotopes or only week 12 in the case of fatty acids. Crabs from these sample dates were used because their biomarker values were generally at equilibrium. Using crabs sampled from other possible combinations of weeks 8, 10, and 12 did not affect our results. We did not include any crabs that died during the experiment as part of our TEF calculations.

All raw data are available through the Gulf of Mexico Research Initiative Information & Data Cooperative (GRIIDC: https://data.gulfresearchinitiative.org, doi: http://doi.org/10.7266/N76971K2).

**Results**

**Growth and survival**

Diet did not have a significant effect on the growth of crabs. For crabs sampled on weeks 8, 10, and 12, those fed clams did not have significantly different growth rate in terms of either carapace width (0.24 mm d-1, n = 12, SD = 0.16) or damp mass (0.18 g d-1, n = 12, SD = 0.2) compared to crabs fed fish (carapace width: 0.19 mm d-1, n = 18, SD = 0.11; damp mass: 0.15 g d-1, n = 18, SD = 0.12) (carapace width: ANOVA F1,27 = 1.031, p = 0.319; damp mass: ANOVA F1,28 = 0.186, p = 0.669 with log x + 1 transformed data) (SI Fig). Despite having similar growth rates, crabs fed a diet of clams had a higher mortality (*Z* = 2.13, *p* = 0.033, 20 of 46 died) than crabs fed fish (9 of 41 died), with most of the mortality in both treatments occurring in the first two weeks of the experiment (S2 Fig). The majority of crabs molted during the experiment, with approximately two-thirds of the sampled crabs (39/58) molting at least once. There was a similar percentage of crabs sampled in both diet treatments that did not molt during the experiment (10/32 and 9/26 for fish and clam diets, respectively).

**Stable isotopes**

The two diet items differed in their δ13C values with fish having a δ13C of -16.673 (SD = 0.277, n = 9) and clams having a slightly lower δ13C of -17.525 (SD = 0.242, n = 6) (ANOVA F1,13 = 37.42, p < 0.001, S3a Fig). Fish had a higher δ15N of 14.419 (SD = 0.210, n = 9), compared to the clams’ δ15N value of 11.122 (SD = 0.277, n = 6) (ANOVA F1,13 = 39.15, p < 0.001, S3b Fig). In general, crabs sampled at the beginning of the experiment (t = 0) had lower isotopic values than their diet items (Fig 1) and showed an overall increase in isotopic values for all diets and all tissues over the course of the 12-week experiment (Fig 1). For most diets and tissues, isotopic values reached equilibrium after approximately 6 or 8 weeks (Fig 1).

**Fig 1. a) δ13C and b) δ15N stable isotope values of claw muscle and hepatopancreas of *Callinectes sapidus* fed clams or fish for 12 weeks in the laboratory.** Error bars represent one SD. Blue box indicates the range of values for the diet items with the mean value indicated by the dashed line (n = 6 to 9 replicates). The numerical values on each plot are the average and standard deviation of the discrimination factor (i.e. the SI TEF) for crabs sampled on weeks 8, 10, and 12.

Stable isotope TEFs (i.e. discrimination factors) were calculated by combining all crabs sampled at weeks 8, 10, and 12. Neither diet nor tissue type influenced δ13C discrimination factors which were all near zero (two-way ANOVA, S1 Table, Fig 1a). On the other hand, δ15N discrimination factors in both tissues were near zero for crabs fed fish, but higher in crabs fed clams (Fig 1b). A two-way ANOVA revealed a significant effect of tissue and a significant interaction of diet and tissue on the δ15N discrimination factor (S2 Table). The highest discrimination factor for δ15N was seen in the claw muscle of crabs fed clams (Δ15N = 1.598 ‰, SD = 0.595).

**Effect of lipid extraction on stable isotope values**

Throughout the text, we report stable isotope values determined following the extraction of fatty acids. From paired samples we found lipid extraction decreased δ15N values by 0.099 to 0.454 ‰ and decreased δ13C values by 0.594 to 2.630 ‰ depending on the species or tissue of the sample (S3 Table).

**Lipid content**

There was no difference between lipid content of the fish diet (1.55%, SD = 0.65, n = 6) and clams (0.95%, SD = 0.55, n = 6) (ANOVA F1,10 = 2.982, p = 0.115). Crab hepatopancreas started at a lipid content of 3.77% (SD = 2.18, n = 22) and increased during the course of the experiment on both diets (Fig 2). The claw muscle of crabs started at a lower lipid content (1.38%, SD = 2.35, n = 22) and stayed relatively constant throughout the experiment on both diets (Fig 2).

**Fig 2. Lipid content (% dry mass) of claw muscle and hepatopancreas of *Callinectes sapidus* fed clams or fish for 12 weeks in the laboratory.** Error bars represent one SD. Blue box indicates the range of lipid content for the diet items with the mean value indicated by the dashed line (n = 6 to 9 replicates).

**Fatty acids**

ANOSIM indicated differences in fatty acid profiles of the two diet items (R = 0.6495, p = 0.005). Some of the most abundant fatty acids in both diet items included 16:0, 18:0, 18:1n-9-cis, 20:5n-3, and 22:6n-3 although the specific proportions differed in the two diets (Table 1, S4 Fig). There were also significant differences among the hepatopancreas of crabs sampled at the beginning of the experiment (t = 0) and crabs fed either diet for 12 weeks (ANOSIM, R = 0.6465, p = 0.001). Similarly, the claw muscle of crabs at week 0 and week 12 crabs on either diet showed differences in the multivariate fatty acid profile (ANOSIM, R = 0.6169, p = 0.001). NMDS plots show that the fatty acid profile of the hepatopancreas and claw muscle of crabs generally diverged from initial conditions and , but did completely overlap with the diet item (Fig 3). The movements in multidimensional spaced tended to be greater in the first 6 to 8 weeks of the experiment, and then diminished, suggesting that the fatty acid profile of the consumers was equilibrating.

**Fig 3. Non-metric multi-dimensional scaling (NMDS) plots of fatty acid profiles in a) claw muscle and b) hepatopancreas of *Callinectes sapidus*.** All points are centroids of n = 3-22 samples. Black points indicate initial crab values. Connected points are the mean values of crabs sampled on weeks 2, 4, 6, 8, 10, and 12 with red indicating crabs fed a fish diet and blue indicating crabs on a clam diet. Values of diet items are represented as unconnected points.

**Table 1. Fatty acid profile and lipid content (% dry mass) of diet items and claw muscle and hepatopancreas of *Callinectes sapidus*.**

Initial crabs and crabs fed fish or clams for 12 weeks in the laboratory. Average value and one SD.

Fatty acid TEFs (i.e. calibration coefficients) were calculated based on crabs sampled at week 12. In general, the pattern of calibration coefficients appeared to be mostly influenced by the tissue type, rather than diet (Fig 4). In most cases, the calibration coefficients for fatty acids were close to 1, with some exceptions (Figs 4 and 5, S5 Fig). Some of the fatty acids that typically had exceptionally high calibration coefficients across tissue types and diets include the saturates 21:0, 22:0, and 23:0, and the polyunsaturates 18:2n-6-cis and 18:4n-3-cis (Figs 4 and 5). Some of the fatty acids that typically had exceptionally low calibration coefficients across tissue types and diets include the polyunsaturates 20:2n-11,14-cis, 20:4n-3, and 22:2 (Figs 4 and 5).

**Fig 4. Fatty acid calibration coefficients for hepatopancreas and claw muscle of *Callinectes sapidus* fed clam or fish diets for 12 weeks in the laboratory.** Error bars represent one SD. Dashed, grey line indicates a value of one. Coefficients are plotted on a log scale.

**Fig 5. Average fatty acid concentration for hepatopancreas and claw muscle of *Callinectes sapidus* fed clam or fish diets for 12 weeks in the laboratory.** Dashed line indicates a one-to-one relationship or a calibration coefficient of 1.0. Fatty acids with calibration coefficients > 2.0 or < 0.5 are labeled.

**Discussion**

**Stable isotopes**

Given two isotopically unique diet items we found that isotopic values of blue crabs resembled their diet after six to eight weeks of controlled feeding in the laboratory. Therefore, field studies making dietary inference from wild-caught crabs should expect δ13C and δ15N values to represent a 1.5 to 2.0 month period of dietary incorporation. δ13C discrimination factors were near zero in all tissues, whereas δ15N discrimination factors were influenced by diet. The δ15N enriched fish diet resulted in lower discrimination factors (i.e., near zero), than the δ15N depleted clam diet. This influence of dietary δ15N on δ15N discrimination factors has been reported by Hussey et al. [16] who found a negative relationship between these two values in a meta-analysis of published studies, but without offering a mechanistic explanation for this phenomenon. Our minimum and maximum δ15N discrimination values of -0.5789 (SD = 1.026) and 1.598 (SD = 0.595), provides further evidence against the once canonical, but now highly-disputed, assumption that nitrogen isotopes fractionate 3.4 ‰ with each trophic level [16].

Our study is not the first to estimate SI TEFs in blue crabs, but builds on previous results by being the first estimate of these parameters in adult blue crabs and by making these estimates in specific tissues (i.e., not whole body samples). Fantle et al. [27] and Dittel et al. [28] have previously estimated these parameters in the early life stages of this species. Both studies fed newly metamorphosed individuals one of six diets spanning primary production sources from phytoplankton to benthic algae, intermediate consumers (snails, brine shrimp), as well as detritus. At the end of the twenty-one day experiments, δ13C TEFs ranged from -3.40 to 1.00 and δ15N TEFs ranged from 0.10 to 3.20 depending on the diet item [27-28]. Our results fall within the range of these previously reported values. Interestingly, Dittel et al. [28] appears to also provide evidence for a negative relationship between dietary δ15N and δ15N discrimination factors as observed in our study and elsewhere [16].

**Fatty acids and lipid content**

After 12 weeks of providing blue crabs with two diets of differing FA profiles, the FAs of claw muscle and hepatopancreas in blue crabs began to equilibrate and shift towards those of their diets, but did not entirely match them. While it is more complicated to interpret the equilibration of an entire multivariate FA profile, rather than just single SI values, it appears the majority of the change in consumer FA profiles occurred after approximately six to eight weeks (Fig 3). Therefore, it appears that SIs and FAs provide a similar time frame of dietary information in blue crab of 1.5 to 2.0 months. This information should be relevant for applications of both biomarker types to field studies of wild-caught crabs. Although not definitive, our results may suggest that the metabolically more active hepatopancreas may indeed have a faster turnover rate than claw muscle, at least for FAs. The multivariate ordination (i.e., NMDS) plots suggests that the hepatopancreas moves towards the dietary FA value at a faster rate than claw muscle.

Most FA calibration coefficients were near 1.0, meaning the consumer tissue FA proportion closely resembled that of the diet at the end of the 12-week experiment. Interestingly, FAs of all classes had examples of exceptionally high calibration coefficients (possibly indicating *de novo* synthesis) including saturates (21:0, 22:0, and 23:0), and polyunsaturates (18:2n-6-cis and 18:4n-3-cis).

To date, there have been very few studies that allow for the calculation of FA TEFs in marine crabs and none in this species. Copeman et al. [39] and Hall et al. [40] estimated FA TEFs in non-Portunid crabs (the Anomuran *Paralithoides platypus* and the Graspid *Parasesarma erythodactyla*), and thus, may be less relevant to dietary inference in the blue crab. The Portunid crab, *Portunus pelagicus,* was found to have high calibration coefficients for 22:6n-3 and low values for 14:0, 18:0, and 22:5n-3 when fed a diet of Graspid crabs [40]. These FAs did not have exceptionally high TEFs in this study. As in this study, the calibration coefficient for 18:2n-6 in *P. pelagicus* was also relatively high (1.73), despite having a diet much higher in 18:2n-6 than in our study (5.17% compared to <0.01%). This result suggests that Portunid crabs may, in fact, be able to synthesize 18:2n-6. A previous study using acetate injection to trace fatty acid synthesis found evidence for *de novo* synthesis of 18:1n-9 (Oleic acid) and 20:5n:3 (EPA) and strictly dietary origins of linoleic (18:2n-6) and alpha-linoleic acids (18:3n-3) in the related *Portunus trituberculatus* (Portunidae) [41].

One difficulty of interpreting calibration coefficients, is that FAs with trace amounts in the diet frequently result in extremely high calibration coefficients. For example, 18:2n-6-cis was less than 0.01% of the fish FA profile, but was 0.71% in the claw muscle of crabs at the end of the experiment, resulting in a calibration coefficient of 141.29. Caution should be used in applying calibration coefficients for trace FAs like this, where even small changes or minor variation can overwhelm the small values. Another difficulty in interpreting calibration coefficients lies in making inference about the physiological mechanism occurring in the consumer. Since FA values are expressed as a percent of the normalized area under the chromatogram, they do not represent an absolute concentration, like in the case of SIs. Therefore, a decrease in one FA can result in an increase in the percentage of another FA, without a true increase in its concentration. Therefore, it is difficult to interpret all calibration coefficients greater than 1.0 as evidence of *de novo* synthesis and all calibration coefficients less than 1.0 as evidence of metabolism.

**Future directions and conclusions**

Although our study is an important step in understanding SI and FA TEFs in the blue crab, there are many other variables that may influence the metabolism, deposition, or synthesis of these biomarkers. Future studies should examine the effect of covariates such as temperature, life stage, salinity, and diet quality on the physiology of these biomarkers, allowing more robust inference from trophic mixing models. For example, diet quality (e.g., δ15N, total lipid content) has been shown to influence FA TEFs [e.g., 42]. Although the diet items used in this study were readily distinguishable in terms of isotopic values and multivariate FA profiles, both the clam and fish had similar total lipid content. This nutritional similarity prevents us from making inference about the effect of total dietary lipid content on blue crab TEFs.

Our 12-week laboratory feeding study provides estimates of trophic enrichment factors for both stable isotopes and fatty acids in the blue crab*.* This work has resulted in TEF values for δ13C and δ15N SIs and 37 unique FAs in two crab tissues under two nutritionally unique diet regimes. Making simultaneous estimates of both classes of trophic biomarkers addresses the parameter needs of a new class of mixing models (Neubauer and Jensen 2015) that uses both SI and FA. New methodologies may eliminate the need for controlled feeding studies to estimate fatty acid trophic enrichment factors, but have not been widely adopted yet (Bromaghin et al. 2017). Although we may have only scratched the surface of covariates that can potentially influence TEF values in this common marine crab, this work will allow us to begin to have greater confidence in the inference from biomarker mixing models that are applied to blue crabs and related species.

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**Supporting Information**

**S1 Table. Two-way ANOVA results for the effect of diet and tissue on δ13C discrimination factors in *Callinectes sapidus****.*

**S2 Table. Two-way ANOVA results for the effect of diet and tissue on δ15N discrimination factors in *Callinectes sapidus.***

**S3 Table. Effect of lipid extraction on stable isotope values.** The Δ value is the difference between the SI values of paired (i.e. duplicate) lipid-extracted and non-lipid-extracted samples.

**S1 Fig. Boxplots of growth rate based on a) carapace width and b) damp mass of *Callinectes sapidus.***Crabs were sampled on weeks 8, 10, and 12 on each of two experimental diets.

**S2 Fig. Cumulative proportion of *Callinectes sapidus* that died during the course of the experiment on each of the two experimental diets.**

**S3 Fig. Average a) δ13C and b) δ15N stable isotope values of diet items.** Points are average of 6 or 9 replicates for clam and fish, respectively. Error bars represent one SD.

**S4 Fig. Fatty acid profiles of fish and clam diet items.** Error bars represent one SD.

**S5 Fig. Histogram of fatty acid calibration coefficients for hepatopancreas and claw muscle of *Callinectes sapidus* fed clam or fish diets for 12 weeks in the laboratory.** Dashed line indicates a value of one.