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Lipid Correction for Carbon Stable Isotope Analysis of Yellowfin Tuna

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Abstract: Carbon stable isotopes ($\delta^{13}C$) are widely used in ecological studies to understand diet, food web dynamics, and movements of marine fishes. Still, $\delta^{13}C$ is influenced by lipid content and often requires chemical extraction or mathematical correction. Here, we developed a species-specific mathematical lipid correction for white muscle tissue of yellowfin tuna (*Thunnus albacares*), a highly migratory finfish of considerable economic and ecological value. Lipid extraction was conducted on yellowfin tuna white muscle tissue (C:N range: 2.96–6.49), and both linear and non-linear lipid correction models for $\delta^{13}C$ were fitted and assessed. Lipid extraction increased $\delta^{13}C$, and to a lesser extent, $\delta^{15}N$ values in yellowfin tuna white muscle tissue, but had no effect on $\delta^{34}S$. Both non-linear models provided better fits to the data than the linear model, suggesting an asymptotic relationship between C:N and $\Delta\delta^{13}C$. Results support the growing body of evidence that C:N ratios can be used to predict lipid corrected $\delta^{13}C$ and highlight the value of mathematical correction approaches. We provide species-specific parameter estimates that can be used for lipid correction of white muscle tissue for $\delta^{13}C$ analysis in yellowfin tuna and similar species for which species-specific models have yet to be developed.

Keywords: lipid extraction; trophic ecology; lipid normalization; *Thunnus albacares*; Scombridae; nitrogen; sulfur; $\delta^{13}C$; $\delta^{15}N$; $\delta^{34}S$

Key Contribution: Lipid extraction increased $\delta^{13}C$ values in yellowfin tuna white muscle tissue, particularly tissues with C:N > 3.5. Species-specific parameters for lipid correction models are provided for use in stable isotope studies.

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1. Introduction

Stable isotope analysis is a frequently utilized tool for understanding foraging behavior, food web dynamics, and habitat shifts in marine organisms [1–3]. Carbon stable isotopes ($\delta^{13}C$) are among the most widely applied in fish ecology [4,5], and are often used to trace sources of primary production in marine food webs because there is little fractionation between trophic steps [6]. Still, a common concern with $\delta^{13}C$ analysis is the fact that lipids tend to have more negative $\delta^{13}C$ values than other compounds (e.g., protein) [7]. As a result, interindividual variation in lipid content can affect bulk carbon isotope values (high lipid content leads to more negative values), leading to incorrect interpretation of trophic pathways and/or movements [8,9].

A variety of lipid corrections have been developed to reduce variation in $\delta^{13}C$ values caused by interindividual variability in lipid content [9,10]. The most straightforward way to address high lipid content is to directly extract lipids (i.e., lipid extraction) from fish tissue samples prior to stable isotope analysis. However, this approach has other consequences, as lipid extraction can alter isotope values of other elements of interest, such as nitrogen ($\delta^{15}N$) [11,12]. Alternatively, a mathematical lipid correction can be applied [9,13]. Such corrections are typically based on lipid extraction experiments from which the relationship

between carbon and nitrogen (C:N) ratios and the difference between bulk carbon and lipid-free carbon (after lipid extraction) can be modeled [10]. The resulting relationship can then be used to estimate lipid-free $\delta^{13}C$ based on the C:N ratio while allowing lipids to be incorporated in other isotope analyses (e.g., $\delta^{15}N$) [9]. While mathematical corrections are widely used, lipid relationships vary among species, and species-specific lipid correction equations are unavailable for many marine fishes [9,11,12].

Here we conduct a lipid extraction experiment to develop a mathematical lipid correction equation for yellowfin tuna (*Thunnus albacares*) white muscle tissue that can be applied to future stable isotope analyses. Yellowfin tuna is a circumtropical, highly migratory predator and is an important component of open ocean ecosystems, supporting economically valuable fisheries around the world. Despite considerable interest in yellowfin tuna dietary studies, a mathematical lipid correction has not yet been developed for the species. The aim of this study was to evaluate linear and non-linear approaches to characterize the relationship between bulk C:N ratios and the change in $\delta^{13}C$ values after lipid extraction and develop a mathematical lipid correction equation for yellowfin tuna white muscle tissue.

2. Materials and Methods

Yellowfin tuna (n = 240) were sampled from recreational charter landings in the northern Gulf of Mexico in 2019 and 2020. Epaxial white muscle tissue (5 cm^3) anterior to the dorsal fin was removed from each individual and frozen at −20 °C. Individual tissue samples were divided into two aliquots and freeze-dried for 48 h, after which one aliquot was prepared for stable isotope analysis without extraction, while the other aliquot was set aside for the lipid extraction procedure. Samples immediately prepared for stable isotope analysis were homogenized using a mortar and pestle, weighed (1.5 ± 0.025 milligrams), complimented with a combustion catalyst (vanadium pentoxide, 3.0 ± 0.025 milligrams), and loaded into a 5 × 9-mm tin capsule. Stable isotope analysis was performed using an elemental analyzer interfaced with a Thermo Scientific (Waltham, MA, USA) Delta V Advantage continuous flow isotope ratio mass spectrometer (EA-IRMS) at Louisiana State University. We quantified stable isotopes of carbon, nitrogen, and sulfur, given that these isotopes are among the most commonly applied in fish ecology studies [14]. Stable isotope values were reported in delta notation (δ) and per mil units (‰) relative to the international measurement standards Vienna Pee Dee Belemnite (for carbon), atmospheric N_2 (for nitrogen), and Vienna Canyon Diablo troilite (for sulfur), using the following equation:

$$\delta^{13}C,\ \delta^{15}N,\ \delta^{34}S(‰) = \left(\frac{R_{sample}}{R_{standard}} - 1\right) \times 1000 \quad (1)$$

where R_{sample} and $R_{standard}$ represent the ratio of heavy to light isotopes in the sample and the standard, respectively. Sample precision was ±0.1‰ for $\delta^{13}C$, ±0.2‰ for $\delta^{15}N$, and ±0.3‰ for $\delta^{34}S$. Carbon: nitrogen (C:N) ratios were expressed as the % carbon relative to the % nitrogen (by weight) of a sample based on uncorrected percentage element data.

After stable isotope analysis of samples from the first aliquot, C:N ratios were examined as a proxy for the presence of lipid in yellowfin tuna white muscle tissue [10]. Lipid extraction was then performed on 36 samples from the second aliquot that were systematically chosen to represent the range of C:N values (2.96–6.49) observed in samples from the first aliquot [15]. The extraction of lipid from white muscle tissue followed a modified protocol outlined by Kim and Koch [16], using a 2:1 chloroform: methanol solution as the solvent instead of petroleum ether [9]. Samples were weighed to 350 mg, placed in glass scintillation vials with 8 mL of 2:1 chloroform: methanol solution, and sonicated for 15 min in a water bath sonicator. The solution was then decanted, and samples were rinsed by sonicating for 15 min in 8 mL of deionized water. The deionized water was decanted and the entire procedure of sonication in chloroform: methanol solution and rinse in deionized water was then repeated. Finally, the lipid extracted samples were oven dried for 24 h at

50 °C and prepared for stable isotope analysis following the same procedure described earlier for the untreated samples.

Differences in $\delta^{13}C$, $\delta^{15}N$, and $\delta^{34}S$ between treated and untreated samples were examined using paired *t*-tests ($\alpha = 0.05$). Yellowfin tuna C:N ratios were plotted against the difference in $\delta^{13}C$ between treated and untreated samples ($\Delta\delta^{13}C$), expressed as:

$$\Delta\delta^{13}C = \delta^{13}C_{\text{treated}}\ (‰) - \delta^{13}C_{\text{untreated}}\ (‰), \quad (2)$$

and three models were evaluated for best fit as a lipid correction for yellowfin tuna. First, a linear model was fit to the yellowfin tuna data [10]. However, because relationships between C:N ratios and $\Delta\delta^{13}C$ are not always linear, non-linear least squares was used to solve for two non-linear approaches used by Logan et al. [9] for bluefin tuna (*Thunnus thynnus*). The first was a three-parameter asymptotic model (non-linear Equation (1)) based on the equation described by Logan et al. [9]:

$$\text{Non} - \text{linear Equation } (1):\ \Delta\delta^{13}C = \frac{a\ *\ C:N + b}{C:N + c} \quad (3)$$

where *a* corresponds to the y-asymptote, $-b/a$ corresponds to the x-intercept (C:N ratio when lipid free), and b/c is equal to the y-intercept (C:N = 0). The second non-linear equation was a two-parameter model based on the mass balance equation of Fry [17] expressed as:

$$\text{Non} - \text{linear Equation } (2):\ \Delta\delta^{13}C = P - \frac{P\ *\ F}{C:N} \quad (4)$$

where *P* corresponds to the $\delta^{13}C$ discrimination between protein and lipid and *F* corresponds to the C:N ratio when lipid free [9]. All models were fit to our yellowfin tuna data to develop species-specific parameters that could be used for future studies on yellowfin tuna and similar species. Models were evaluated using Akaike Information Criterion (AIC) and developed and assessed using the R Statistical Programming Environment [18].

3. Results

Yellowfin tuna C:N ratios ranged from 2.96 to 6.49 with a mean (±SE) of 3.93 ± 0.15. In general, higher C:N ratios were observed for larger individuals (Table S1). Lipid extraction of white muscle tissue resulted in a significant increase in $\delta^{13}C$ values ranging from 0–3.4‰ with a mean of 1.06 ± 0.18‰ (mean increase ± SE) across all yellowfin tuna samples (paired *t*-test; $p < 0.001$). A significant increase in $\delta^{15}N$ ± (0.33 ± 0.08‰, mean difference ± SE) was also observed (paired *t*-test, $p < 0.001$); however, lipid extraction did not influence $\delta^{34}S$ values (0.14 ± 0.08‰) (paired *t*-test, $p > 0.05$).

All models indicated that C:N was a good predictor of $\Delta\delta^{13}C$ after lipid extraction. The linear model was described by $\Delta\delta^{13}C = 1.16(C:N) - 3.51$, with a residual standard error of 0.32 and an adjusted R^2 of 0.92 (Figure 1A). Standard errors for parameter estimates were 0.06 for the slope and 0.24 for the y-intercept. Still, AIC indicated the non-linear models provided a better fit to describe the relationship between C:N and $\Delta\delta^{13}C$ for yellowfin tuna (Figure 1). Non-linear equation 1 had the lowest AIC value and residual standard error (AIC = 12.10, RSE = 0.21) compared to non-linear equation 2 (AIC = 13.84, RSE = 0.28) and the linear equation (AIC= 23.21). The difference in AIC between the two non-linear models was considered negligible [19]. Parameter estimates for non-linear Equation (1) were $a = 9.36 \pm 2.31$ (SE), $b = -29.36 \pm 7.11$, and $c = 2.18 \pm 1.88$ (Table 1). In contrast, parameter estimates for non-linear equation 2 were $P = 6.64 \pm 0.25$ (SE) and $F = 3.16 \pm 0.03$ (Table 1). The x-intercept in all models represented the C:N ratio below which $\delta^{13}C$ did not increase after lipid extraction (C:N ratio of lipid free sample), and was similar between non-linear Equation (1) ($-b/a = 3.14$) and non-linear Equation (2) ($F = 3.16$) but was slightly lower for the linear model (3.03).

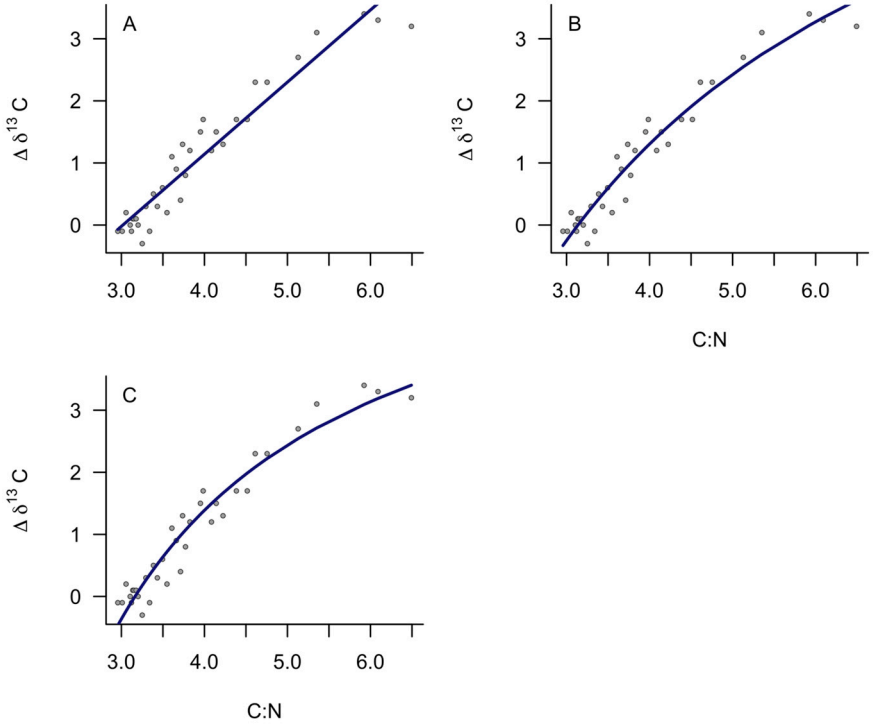

Figure 1. Model fits showing the relationship between carbon: nitrogen ratios and the change in carbon isotope after lipid extraction for yellowfin tuna (n = 36) across a range of C:N. Models represent (**A**) Linear, (**B**) Non-linear Equation (1), and (**C**) Non-linear Equation (2).

Table 1. Lipid correction equations for yellowfin tuna with parameter estimates and standard errors (SE). The term $\delta^{13}C_{Bulk}$ refers to untreated $\delta^{13}C$ value, while $\delta^{13}C_{Lipid\text{-}free}$ refers to $\delta^{13}C$ value after lipid correction. C:N refers to the carbon: nitrogen ratio of untreated $\delta^{13}C$.

Model	Lipid Correction Equation	Parameter Estimates (SE)
Linear	$\delta^{13}C_{Lipid-free} = \delta^{13}C_{Bulk} + (1.16 * C:N - 3.51)$	a = 1.161 (0.059) b = −3.505 (0.236)
Non-linear Equation (1)	$\delta^{13}C_{Lipid-free} = \delta^{13}C_{Bulk} + \left(\frac{9.356 * C:N - 29.359}{C:N + 2.181}\right)$	a = 9.356 (2.310) b = −29.359 (7.108) c = 2.181 (1.875)
Non-linear Equation (2)	$\delta^{13}C_{Lipid-free} = \delta^{13}C_{Bulk} + \left(6.637 - \frac{6.637 * 3.164}{C:N}\right)$	P = 6.637 (0.249) F = 3.164 (0.031)

4. Discussion

Lipid extraction of yellowfin tuna white muscle tissue resulted in a substantial increase in $\delta^{13}C$ values, suggesting that lipid correction (e.g., either extraction or mathematical) may be necessary for $\delta^{13}C$ stable isotope analysis. While it is not uncommon for fish white muscle tissue to have low lipid content, large pelagic predators such as tunas, swordfish, and sharks often have higher lipid content in white muscle and require lipid correction for stable isotope analysis [9,20,21]. Interestingly, the highest C:N ratios were observed in large yellowfin tuna that were captured during winter or early spring. While variance in lipid content can reflect individual or spatio-temporal differences in diet, similar seasonal trends in C:N have been reported for bluefin tuna and albacore (*Thunnus alalunga*) in the north Atlantic, with increased lipid content during cooler months attributed to reduced allocation to egg production during winter [22]. It is unclear if a similar mechanism applies to yellowfin tuna in the Gulf of Mexico; however, it is worth noting that approximately 75% of the individuals with C:N ratios greater than 4.0 were females. Finally, lipid extraction resulted in small, but significant alteration of $\delta^{15}N$ values in yellowfin tuna, which is in agreement with previous studies that have reported more substantial changes in $\delta^{15}N$ after

lipid extraction [23]. In contrast, our results indicate that extraction has little influence on $\delta^{34}S$. Still, these results suggest that a mathematical correction may be preferred to extraction to correct for lipids without altering interpretation of $\delta^{15}N$ or $\delta^{34}S$.

Similar to other marine fishes, a strong predictive relationship was observed between the C:N ratio and $\Delta\delta^{13}C$ for yellowfin tuna white muscle tissue after lipid extraction [24,25]. The finding that both non-linear approaches to lipid correction provided better fits relative to the linear approach is consistent with several recent studies suggesting that the relationship between C:N and $\Delta\delta^{13}C$ is asymptotic in tunas and other large predators [9,12,24]. Linear approaches are also common for lipid correction in marine fishes; however, because the isotopic discrimination in $\delta^{13}C$ between lipid and protein is believed to be between 6–7‰ [8], it might be expected that the rate of change in $\delta^{13}C$ will approach an asymptote at higher C:N ratios [10,25]. Thus, despite the fact that the linear model provided a good fit in the current study, our results support the notion that a non-linear correction is likely to perform better over a wide range of C:N ratios [9]. The mass balance equation (non-linear Equation (2), [17]) is often recommended for lipid normalization of marine fish muscle tissue [9,24,26] however, non-linear Equation (1) provided the best fit in the current study. Still, differences between both non-linear equations were negligible [19], suggesting that either approach would be appropriate for yellowfin tuna.

Lipid extraction prior to stable isotope analysis is the subject of some debate, and while lipid correction is generally recommended when C:N > 3.5 [10]; other studies have found this reference to be unreliable, as relationships between C:N and lipid content may vary by species, tissue, or trophic grouping [12,27]. Our best fit model indicated that yellowfin tuna white muscle tissue with C:N ratios > 3.14, could be expected to undergo some measure of alteration in $\delta^{13}C$ after lipid extraction and could therefore be corrected. Still, differences in $\delta^{13}C$ after extraction did not exceed 0.3‰ (mean difference = 0.02) when C:N ratios < 3.3, or 0.5‰ (mean difference = 0.1) when C:N ratios < 3.5. Thus, it appears that in practice, lipid extraction will have little influence on $\delta^{13}C$ values for yellowfin tuna white muscle tissue with C:N ratios < 3.5, corroborating the general recommendation of Post et al. [10].

Stable isotope approaches are increasingly incorporated in food web studies to better understand trophic linkages supporting pelagic fish populations [28–30]. Our findings suggest lipid extraction or correction is needed for analysis of carbon stable isotopes in yellowfin tuna when lipid content (C:N ratio) is elevated, and highlight the benefits of mathematical correction approaches which reduce time and costs of lipid extraction while avoiding the potential negative impacts of lipid removal on $\delta^{15}N$ [9]. While several general (multi-taxa) models have been developed for lipid correction [9,10], species- and tissue-specific lipid correction models generally perform best when available [9,12,26]. Thus, the species-specific parameter estimates for lipid correction in yellowfin tuna white muscle tissue presented here can be applied to other $\delta^{13}C$ studies within the general range of C:N ratios included in our models (~2.9–6.5). While species-specific approaches are certainly preferred when available, model parameter estimates presented here may also prove useful for lipid correction of white muscle tissue in other tropical scombrids for which species-specific corrections have yet to be developed.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fishes8090446/s1, Table S1: Summary data for yellowfin tuna samples selected for lipid extraction.

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Data Availability Statement: Data from this study can be accessed from the authors upon reasonable request.

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Conflicts of Interest: The authors declare no conflict of interest.

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