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Experimental validation confirms a carbon stable isotope lipid normalization procedure for Pacific salmon

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ABSTRACT: Carbon stable isotope analysis is an important tool in studies of fish ecology. Studies using this tool must account for the effect of lipids present in fish tissue on carbon stable isotope ($\delta^{13}C$) values. Simple correction equations have been developed to correct for this effect. For taxa with high fat content, choosing an accurate correction equation can have a significant impact on results. Pacific salmon (*Oncorhynchus* spp.) are a lipid-rich genus. Their broad marine distribution and ecological, cultural, and commercial importance make them prime candidates for stable isotope analysis. To determine both an accurate lipid correction equation for Pacific salmon $\delta^{13}C$ values, and the effect of lipid extraction on bulk nitrogen isotope ($\delta^{15}N$) values, we performed pairwise isotope analysis on lipid-extracted and untreated muscle samples from 68 Chinook salmon *O. tshawytscha* spanning a size range of 165–750 mm and C:N values of 2.96–14.25. We compared the fit of existing $\delta^{13}C$ lipid correction equations from 3 previously published models to our data, and optimized the top performing model using a leave-one-out cross validation. The model of Kiljunen et al. (2006; https://doi.org/10.1111/j.1365-2664.2006.01224.x) performed the best (mean squared error: 0.22, r^2: 0.91), while the optimized model only slightly improved on it (MSE: 0.20, r^2: 0.93). For $\delta^{15}N$, we determined that Chinook $\delta^{15}N$ values significantly increased by 0.6‰ following lipid extraction. Our results confirm a lipid normalization procedure that is broadly applicable to Pacific salmon, and supports streamlined analysis of $\delta^{13}C$ and $\delta^{15}N$ from a single untreated muscle tissue sample.

KEY WORDS: Chinook salmon · *Oncorhynchus tshawytscha* · C:N ratio · Correction equation

1. INTRODUCTION

Carbon stable isotope analysis (SIA) has become an important tool in studies of fish trophodynamics (Kelly et al. 2019), habitat use (Fry 2002) and distribution (Espinasse et al. 2020). Carbon stable isotope values, denoted $\delta^{13}C$, represent the ratio of heavy (^{13}C) to light (^{12}C) carbon isotopes in a sample relative to a standard. Studies have established that $\delta^{13}C$ values increase by roughly 0.5–1‰ with each trophic transfer due to enrichment of the heavier ^{13}C isotope, such that the $\delta^{13}C$ value of a consumer represents an assimilation of the $\delta^{13}C$ values of its diet sources (DeNiro & Epstein 1978, Pinnegar & Polunin 1999). The assimilated nature of stable isotope signatures makes them valuable

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tools to research organisms beyond the reach of traditional diet studies.

SIAs that utilize carbon isotopes must acknowledge a major caveat with the data, namely that $\delta^{13}C$ values can be influenced by lipids present in a sample (McConnaughey & McRoy 1979). Lipids are depleted in ^{13}C relative to muscle tissue, and therefore $\delta^{13}C$ values will be lower in tissue with high lipid content than in tissue with low lipid content (McConnaughey & McRoy 1979). Variation in lipid content can cause variation in $\delta^{13}C$ values larger than the expected 0.5–1‰ difference between trophic levels, which can subject results to misinterpretation (Kiljunen et al. 2006).

The most effective way to account for the impact of lipids on $\delta^{13}C$ values is to chemically extract the lipids from the sample before analysis. Unfortunately, lipid removal can impact the nitrogen isotope value ($\delta^{15}N$) of a sample (Pinnegar & Polunin 1999, Logan et al. 2008). This occurs because the lipid extraction procedure also results in the loss of non-lipid compounds that can alter $\delta^{15}N$ values (Sweeting et al. 2006). In SIA, bulk $\delta^{13}C$ and $\delta^{15}N$ values are usually measured in tandem, and this implies that it is necessary to process isotope samples twice, once with a chemical lipid removal and once without, to get accurate measures of both.

To simplify and streamline sampling, many analyses rely on mathematical correction equations to normalize untreated $\delta^{13}C$ values by total lipid content (McConnaughey & McRoy 1979). However, as deriving quantitative estimates of total lipid content can be just as intensive as chemical lipid removal, many studies use C:N ratios of bulk tissue ($C:N_{bulk}$) as a proxy for total lipid content (Post et al. 2007). Lipids are composed of long chains of carbon and most contain no nitrogen; thus, increases in $C:N_{bulk}$ ratios track increases in lipid content (Logan et al. 2008). Among models that normalize $\delta^{13}C$ values, 3 common approaches are applied: linear regression based $C:N_{bulk}$ ratios (e.g. Post et al. 2007), arithmetic mass balance using $C:N_{bulk}$ (e.g. Fry 2002) and a lipid normalization based on $C:N_{bulk}$ originally proposed by McConnaughey & McRoy (1979).

While universal correction models have appeal, both Logan et al. (2008) and Fagan et al. (2011) found that fitting a model to a specific taxon or tissue is more robust than choosing from any of the 3 possible approaches described above. This is particularly important for taxa with high lipid content where the effect of normalization is largest, and an incorrect assumption can bias the interpretation of the data (Kiljunen et al. 2006). Fitting a model to a specific taxon and tissue is an intensive process as it involves pairwise isotope analysis of bulk lipid-extracted tissue and untreated tissue (Logan et al. 2008). It should thus be applied prudently to groups with a high potential for application of SIA.

Pacific salmon (*Oncorhynchus* spp.) are an important genus for such an endeavor. They include several species of high ecological, cultural and commercial importance, and are broadly distributed across the temperate and sub-polar North Pacific, extending into the Arctic (pink *O. gorbuscha* and chum *O. keta*), occurring in increasing numbers in the North Atlantic (pink), and also in the Great Lakes of North America. Their marine life history stage takes them beyond the range of routine surveys that can assess trophic ecology, yet it is in the ocean where most of their growth occurs. SIA could provide new insight into this critical life history stage. Many studies of Pacific salmon have used SIA and relied on generic lipid correction factors to estimate $\delta^{13}C$ values (e.g. Johnson & Schindler 2009). Despite this, none of the commonly used lipid corrections in the literature (e.g. Kiljunen et al. 2006, Post et al. 2007, Hoffman et al. 2015) incorporated Pacific salmon while deriving their equations. Because lipid correction is sensitive to protein-lipid $\delta^{13}C$ discrimination and baseline C:N ratios of lipid-free tissue (Logan et al. 2008), having lipid corrections from a closely related species is preferred for accurate estimation of $\delta^{13}C$ values.

In this study, we sought to develop a lipid-correction model for Pacific salmon $\delta^{13}C$ values using tissue from Chinook salmon *O. tshawytscha*, as this species exhibits the broadest range of lipid values within the genus *Oncorhynchus* (O'Neill et al. 2014). We performed a pairwise isotope analysis on bulk, untreated and lipid-extracted Chinook muscle tissue. Our objectives were (1) to determine the impact of chemical lipid removal of Chinook muscle tissue on $\delta^{15}N$ values and (2) to determine the optimal correction equation to model the effect of lipid content on $\delta^{13}C$ values. To resolve objective 2, we used the $C:N_{bulk}$ ratio as a proxy for lipid content and compared lipid-correction model fits from 3 different approaches from the literature: a linear model, a mass balance model and the lipid normalization model of McConnaughey & McRoy (1979). Finally, we sought to optimize the most accurate model to our data.

2. MATERIALS AND METHODS

2.1. Sample source

Chinook salmon samples (n = 68) used in this study came from 3 sources: 33 fish (165–220 mm fork length [FL]) were from controlled feeding experiments at the Pacific Science Enterprise Centre, West Vancouver, British Columbia (BC), Canada; 7 fish (>600 mm FL) came from Yellow Island aquaculture, Quadra Island, BC; and 28 individuals (600–750 mm FL) were collected from samples opportunistically taken from the Albion test fishery, a Canadian Department of Fisheries and Oceans gill net test fishery operating in Maple Ridge, BC. All salmon were frozen until laboratory processing.

2.2. Sample processing

A sample of dorsal muscle tissue was taken from all specimens. Tissue was oven dried at 50°C for 48 h and then homogenized with a mortar and pestle. A 0.5 mg portion was packaged directly for isotope analysis in tin capsules while another portion was subdivided for lipid extraction. Lipids were extracted using chloroform and methanol following the method of Folch et al. (1957) modified by Post & Parkinson (2001) and Arrington et al. (2006). Briefly, a roughly 0.5 g sample was placed in a test tube, and 8 ml of chloroform and 8 ml of methanol were added. The sample was then heated to 61°C in a water bath until the solution boiled. The sample was allowed to cool to room temperature, and 8 ml of chloroform were added. The sample and solution were then poured over a Whatman filter. The solution was drained and discarded. This process was repeated 3 times or until the solution was clear. Samples were then dried for 24 hours at 50°C. Following this lipid extraction procedure, these samples were also packaged for SIA in tin capsules.

2.3. SIA

SIA was completed at the Great Lakes Institute of Environmental Research, University of Windsor in Windsor, Ontario, Canada. Bulk isotope samples were analyzed for their $\delta^{13}C$ and $\delta^{15}N$ values using a Delta V Advantage Mass spectrometer (Thermo) coupled to a Costech 4010 Elemental Combustion system and a ConFlo IV gas interface. Precision, assessed by the standard deviation of replicate analyses of 4 standards (NIST1577c, internal lab standard [tilapia muscle], USGS 40 and urea; n = 35 for all) was ≤0.2‰ for both $\delta^{15}N$ and $\delta^{13}C$ for all the standards.

2.4. Statistics

All data were processed in R version 4.1.1 (R Core Team 2021).

Slope tests were used to determine if the slope of the relationship between bulk, untreated and lipid-extracted $\delta^{13}C$ and $\delta^{15}N$ values was equal to 1.0. Paired *t*-tests were used to determine whether changes in $\delta^{13}C$ and $\delta^{15}N$ associated with lipid extraction were significant for Chinook muscle.

Three different lipid-correction models from the literature, covering the 3 main approaches, were compared: linear regression (Post et al. 2007), arithmetic mass balance (Hoffman et al. 2015) and the McConnaughey model (Kiljunen et al. 2006).

The linear regression model relates the C:N ratio to the difference between $\delta^{13}C_{Lipid\text{-}extracted}$ and $\delta^{13}C_{Untreated.}$ The model derived by Post et al. (2007) is:

$$\delta^{13}C_{Lipid\text{-}extracted} - \delta^{13}C_{Untreated} = -3.32 + 0.99 \times C{:}N_{Bulk} \quad (1)$$

The arithmetic mass balance equation assumes that a bulk tissue sample is composed of 2 fractions ($f_{Lipid} + f_{Lipid\text{-}free} = 1$). Lipid content can then be estimated from the $C{:}N_{Bulk}$ ratio if the $C{:}N_{Lipid\text{-}free}$ ratio is known ($f_{Lipid} = 1 - C{:}N_{Lipid\text{-}free} / C{:}N_{Bulk}$). From there, the difference between $\delta^{13}C_{Lipid\text{-}extracted}$ and $\delta^{13}C_{Untreated}$ can be calculated as:

$$\delta^{13}C_{Lipid\text{-}extracted} - \delta^{13}C_{Untreated} = (D \times [C{:}N_{Lipid\text{-}free} - C{:}N_{Bulk}]) / C{:}N_{Bulk} \quad (2)$$

where *D* is the lipid discrimination value, i.e. the isotopic difference between protein and lipid, and $C{:}N_{Lipid\text{-}free}$ is an assumed constant. In the mass balance model of Hoffman et al. (2015), these parameters were estimated as −6.5‰ for *D* and 3.5 for the ratio of $C{:}N_{Lipid\text{-}free}$.

The lipid normalization procedure of Kiljunen et al. (2006) is based on the McConnaughey model. It differs from the mass balance model mainly in the method they used to estimate lipid content from C:N ratios. Unlike in the mass balance model, in the McConnaughey model, tissue is not assumed to be divided into 2 fractions but is believed to be 93% proteins and lipids with the rest being carbohydrates. Lipids (*L*) can then be estimated from the equation:

$$L = 93 / 1+ (0.246 \times \text{C:N} - 0.775)^{-1} \quad (3)$$

and $\delta^{13}C_{\text{Lipid-extracted}} - \delta^{13}C_{\text{Untreated}}$ can be estimated from the following equation:

$$\delta^{13}C_{\text{Lipid-extracted}} - \delta^{13}C_{\text{Untreated}} = D \times (I + [3.90/(1 + 287/L)]) \quad (4)$$

In this model, D again represents the lipid discrimination value and was estimated by Kiljunen et al. (2006) as 7.018, whereas I is a constant estimated as 0.048.

For all models, r^2 and mean squared error (MSE) were calculated. Using the R package 'minipak.lm' (version 1.2-1), the top-fitting model was optimized for our data using an nlsLM model with a leave-one-out cross validation (LOOCV) to calculate MSE. A LOOCV splits the data into a training and testing set, using all but 1 datapoint in the training set used to build the model, and repeats for every observation. In doing so, it can provide an unbiased MSE to evaluate model performance.

3. RESULTS

The slope of the regression between $\delta^{15}N_{\text{Untreated}}$ and $\delta^{15}N_{\text{Lipid-extracted}}$ was equal to 1.0 ($p = 0.72$). The slope of the regression between $\delta^{13}C_{\text{Untreated}}$ and $\delta^{13}C_{\text{Lipid-extracted}}$ was <1.0 ($p < 0.0001$). Paired t-tests indicated significant differences between $\delta^{15}N_{\text{Untreated}}$ and $\delta^{15}N_{\text{Lipid-extracted}}$ samples ($p < 0.0001$) and between $\delta^{13}C_{\text{Untreated}}$ and $\delta^{13}C_{\text{Lipid-extracted}}$ samples ($p < 0.0001$). Chinook $\delta^{13}C_{\text{Lipid-extracted}}$ values were lower by an average of 2.5‰ relative to $\delta^{13}C_{\text{Untreated}}$ values but showed a clear curvilinear trend, with $C:N_{\text{Bulk}}$ ratios suggesting that this effect leveled off in tissue with higher fat content (Figs. 1 & 2B). Chinook $\delta^{15}N$ values consistently increased by an average of ~0.6‰ following lipid extraction (Fig. 2A).

In agreement with the visual fit (Fig. 1), the MSE and r^2 of the 3 lipid extraction models from the literature indicated that the correction of Kiljunen et al. (2006) was most accurate, followed by Hoffman et al. (2015) and then Post et al. (2007) (Table 1). Optimization of the Kiljunen et al. (2006) model did not noticeably increase the visual fit or significantly improve the MSE or r^2 (Table 1). Fitting the model with an nlsLM optimization re-estimated the 2 parameters (D and I) as 6.31 and 0.103, respectively.

4. DISCUSSION

Our results demonstrated that Chinook $\delta^{13}C$ and $\delta^{15}N$ values significantly increased following lipid extraction (Fig. 2). $\delta^{15}N$ increased 0.6‰ following lipid extraction, and the slope of the regression between $\delta^{15}N_{\text{Bulk}}$ and $\delta^{15}N_{\text{Lipid-extracted}}$ was equal to 1 indicating that this change in $\delta^{15}N_{\text{Lipid-extracted}}$ was highly consistent. In ecological applications, $\delta^{15}N$ data are often used to determine the trophic level of an organism. $\delta^{15}N$ values are often assumed to increase by 3.4‰ for each trophic transfer (Post 2002), meaning that the 0.6‰ increase observed in our study, though small, could bias the interpretation of trophic level upwards if not taken into consideration.

Our results showed that the lipid correction procedure of Post et al. (2007) is not appropriate for Chinook salmon. The MSE for this model was 2.01, indicating an error far above the <1‰ precision considered acceptable in food web studies (Table 1) (Hoffman et al. 2015). This result was not unex-

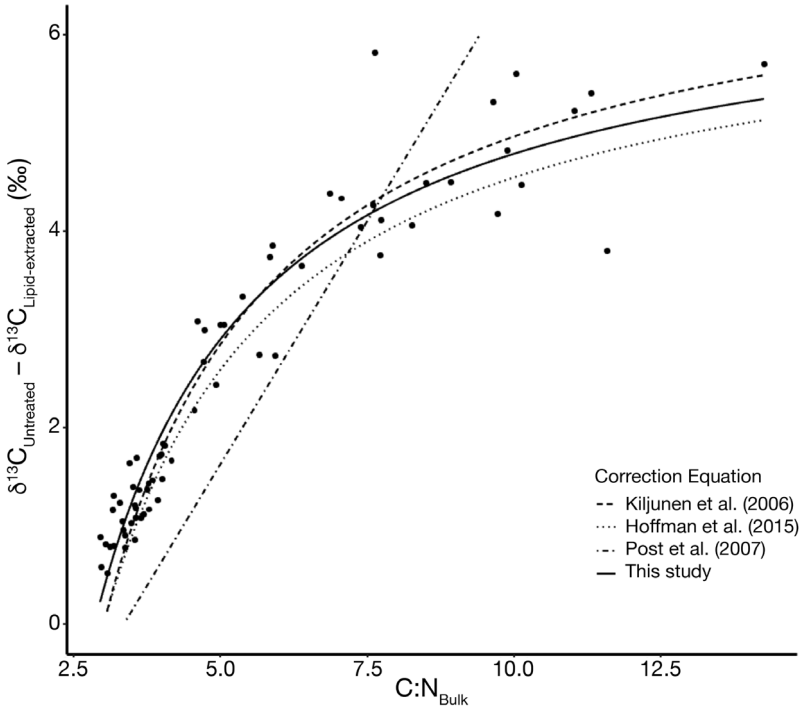

Fig. 1. Fit of the 4 models in this study (see Table 1) to $C:N_{\text{Bulk}}$ values and the difference between untreated $\delta^{13}C$ and lipid-extracted $\delta^{13}C$ values of Chinook salmon

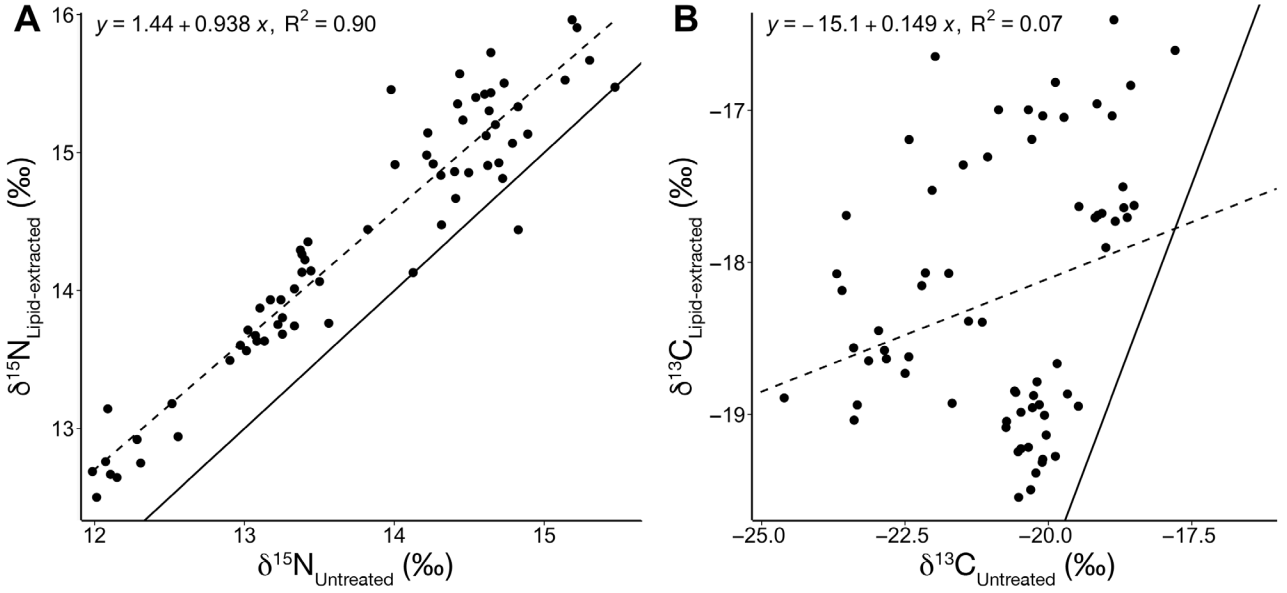

Fig. 2. Relationship between (A) $\delta^{15}N$ and (B) $\delta^{13}C$ untreated and lipid-extracted Chinook muscle tissue. Solid line indicates 1:1 relationship, dashed line indicates best fit

Table 1. Mean squared error (MSE) and r^2 values of the 3 lipid normalization models from the literature and the optimized Kiljunen model for the relationship between the $C:N_{Bulk}$ and the difference between untreated $\delta^{13}C$ and lipid-extracted $\delta^{13}C$ values of Chinook salmon

Model	MSE	r^2
Kiljunen et al. (2006)	0.22	0.91
Hoffman et al. (2015)	1.30	0.63
Post et al. (2007)	2.01	0.24
This study (optimized Kiljunen)	0.20	0.93

pected: the equation of Post et al. (2007) is a simple linear regression, and the asymptotic relationship between the C:N ratio and the difference between $\delta^{13}C_{Lipid\text{-}extracted}$ and $\delta^{13}C_{Untreated}$ has been noted in other studies (Kiljunen et al. 2006, Logan et al. 2008, Hoffman & Sutton 2010, Hoffman et al. 2015). This curvilinear response between $C:N_{bulk}$ and the difference between $\delta^{13}C_{Lipid\text{-}extracted}$ and $\delta^{13}C_{Untreated}$ occurs because the lipid discrimination value (*D*), the isotopic difference between protein and lipid, represents the maximum possible decrease in $\delta^{13}C$ in a sample as lipid content increases and nitrogen is diluted (Hoffman et al. 2015).

Our samples had a wide range of $C:N_{Bulk}$ values, and our analysis showed a curvilinear relationship between $C:N_{Bulk}$ and the difference between $\delta^{13}C_{Lipid\text{-}extracted}$ and $\delta^{13}C_{Untreated}$ (Fig. 1). The results of our model validation established effective lipid correction equations for Chinook salmon, and confirm the effectiveness of the lipid normalization equation of Kiljunen et al. (2006). This lipid normalization equation (Kiljunen et al. 2006), with respect to both its low MSE and high r^2, demonstrated a strong predictive power. Its MSE of 0.22 is well within the precision necessary for food web studies. Our attempts to improve this equation through an nlsLM optimization with LOOCV improved these metrics only by 0.02 for both the MSE and r^2 values. Optimization was likely unnecessary, but both equations demonstrate an effective procedure for correcting for lipids in Chinook bulk isotope analysis without extraction. Our data covered a wide spectrum of Chinook size and lipid content (as demonstrated by the range in $C:N_{Bulk}$ values) and thus support the broad application of these correction equations over the full spectrum of life history for this species.

The robustness of the lipid normalization equation of Kiljunen et al. (2006) may be due to the broad sample set used to create this model. Unlike Hoffman et al. (2015), who used data from 5 species with an average $C:N_{Bulk}$ ratio of 3.8–5.3, the model of Kiljunen et al. (2006) was derived from data encompassing 227 specimens from 14 species with average $C:N_{Bulk}$ ratios ranging between 3.00 and 9.36. This comprehensive analysis may also have been effective for Chinook because it encompassed a diverse group of fish including similar species such as Atlantic salmon *Salmo salar* and others with high lipid content, such as American eel *Anguilla rostrata*.

A reason the equation of Kiljunen et al. (2006), and not the original McConnaughey model, was most applicable is that in the latter study lipids were normalized to a C:N ratio of 4.0. This ratio is likely too high, as both the results of our analysis and that of Kiljunen et al. (2006) indicated that lipid-free muscle tissue in these fish species has a C:N ratio close to 3.0. This same result can likely explain the shortcomings of the mass balance model of Hoffman et al. (2015) investigated in this study. This model was ineffective for Chinook salmon tissue in part because lipids were normalized to a $C:N_{Bulk}$ ratio of 3.5. It seems that if the mass balance model was fit to our data, possibly just by changing the C:N ratio (which sets the *x*-intercept) from 3.5 to a more realistic 3.0, the shift of the equation would cause it also to be highly accurate. This is in agreement with the conclusions of Logan et al. (2008) that fitting a model to a specific taxon and tissue is more relevant than picking between specific approaches.

The lipid discrimination value for the model of Hoffman et al. (2015), set as 6.5‰, may be incorrect for Chinook. Lipid discrimination can vary between tissues, species and populations, and has been estimated between 6 and 7‰ for multiple fish species (McConnaughey & McRoy 1979, Fry 2002, Sweeting et al. 2006, Hoffman et al. 2015). The original McConnaughey model also may have underestimated this parameter, setting it at 6‰, while the more effective model of Kiljunen et al. (2006) derived a value of 7‰. Our analysis re-estimated 2 parameters from the McConnaughey model, *D* and *I* from Eq. (4), as 6.31‰ and 0.103, respectively. As our analysis did not empirically derive new estimates of *D*, we cannot confirm the true isotopic difference between protein and lipid for Chinook.

Pacific salmon are an extraordinarily diverse genus with respect to life history, and lipid content can vary dramatically both within and among species (O'Neill et al. 2014). We have demonstrated that removing lipids to correct $\delta^{13}C$ values can impact $\delta^{15}N$ values. To negate the need for duplicate sampling, the results of our pairwise comparison of bulk, untreated and lipid-extracted Chinook salmon tissue provide support for a single mathematical correction factor, the correction equations of Kiljunen et al. (2006), to account for the impact of lipids (using the $C:N_{Bulk}$ ratio as a proxy) on $\delta^{13}C$ values in this genus and streamline SIA of Pacific salmon tissue. Alternatively, the consistent offset between $\delta^{15}N_{Bulk}$ and $\delta^{15}N_{Lipid\text{-}extracted}$ could be applied to correct $\delta^{15}N$ values measured from lipid-extracted samples.

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