NOTE

**Lipid extraction in stable isotope analyses of juvenile sea turtle skin and muscle**

Thaisa F. Bergamo¹,², Silvina Botta²,³, *, Margareth Copertino¹,²

¹Laboratório de Ecologia Vegetal Costeira, Instituto de Oceanografia, Universidade Federal do Rio Grande - FURG, CP 474, Rio Grande, RS 96203-900, Brazil

²Programa de Pós-Graduação em Oceanografia Biológica, Instituto de Oceanografia, Universidade Federal do Rio Grande - FURG, CP 474, Rio Grande, RS 96203-900, Brazil

³Laboratório de Ecologia e Conservação da Megafauna Marinha, Universidade Federal do Rio Grande - FURG, CP 474, Rio Grande, RS 96203-900, Brazil

**ABSTRACT:** Studies involving various aspects of the biology and ecology of sea turtles have successfully applied stable isotope analysis. In many of these studies, the chemical extraction of ¹³C-depleted lipids of sea turtle tissues has been used as a standard protocol, often without testing whether the time-consuming lipid removal is required. Furthermore, this chemical procedure may unpredictably modify δ¹⁵N values, probably due to the loss of proteins associated with lipid structures, thus reinforcing the need for testing the isotopic consequence of the chemical removal of lipids. This study aimed to evaluate the effects of lipid extraction on skin and muscle C and N isotopic values of juvenile green turtles *Chelonia mydas*. We analyzed paired δ¹³C and δ¹⁵N values from lipid-extracted and non-lipid-extracted samples of skin and muscle of 15 juvenile green turtles. Lipid extraction was performed using a mixture of chloroform and methanol. A significant increase was found in δ¹³C values after lipid extraction for muscle (~0.5‰), but not for skin. C:N ratios were not correlated with the change in δ¹³C values in either tissue. δ¹⁵N values were not affected by lipid extraction in either tissue. The difference found in δ¹³C values between control and lipid-extracted muscle samples may be biologically significant. On the other hand, the lipid extraction of skin samples does not appear to be necessary in the case of juvenile green turtles. This procedure needs to be tested in green turtles in other life stages.

**KEY WORDS:** Green turtle · *Chelonia mydas* · Stable isotope analysis · Carbon · Nitrogen · δ¹³C · δ¹⁵N · Lipid extraction

**INTRODUCTION**

Stable isotope analysis (SIA) of animal tissue carbon (δ¹³C values) and nitrogen (δ¹⁵N values) has been largely used to study the trophic ecology and habitat use of several taxa, including marine megafauna (e.g. seabirds, Cherel et al. 2005; sea turtles, Arthur et al. 2008; sharks, Carlisle et al. 2015; marine mammals, Drago et al. 2015). The method is based on the principle that the isotopic composition in the tissues of consumers reflects the mixture of the isotopic composition present in the different dietary items consumed (DeNiro & Epstein 1978, 1981). The δ¹⁵N values predictably increase with increasing trophic level (DeNiro & Epstein 1981, Minagawa & Wada 1984) and consequently, they are used as indicators of a consumer’s trophic position (Post 2002, McCutchan et al. 2003, Vanderklift...
& Ponsard 2003). On the other hand, δ\(^{13}\)C values vary little along the food chain and are mainly used to infer primary sources in a food web (McCutchan et al. 2003).

In recent years, several studies using SIA have addressed the issue of the effects of lipids on the isotopic composition of tissue carbon (e.g. Lesage et al. 2010, Ruiz-Cooley et al. 2011, Ryan et al. 2012). Due to differences in the biochemical pathways during synthesis, lipids may be depleted in \(^{13}\)C relative to other major constituents, such as proteins (DeNiro & Epstein 1977). Such differences among protein and lipid metabolism (Martínez del Río et al. 2009) can bias the interpretation of stable isotope values and result in misleading diet reconstructions, especially when based on tissues with a significant amount of fat (Ricca et al. 2007). Therefore, lipids are usually removed from tissues by either chemical lipid extraction or mathematical correction using species- and tissue-specific equations (e.g. Logan et al. 2008, Elliott et al. 2014). Chemical extraction of lipids is generally effective, yielding isotopic values of carbon that can be considered free of the influence of these biomolecules (Post et al. 2007, Logan et al. 2008, Medeiros et al. 2015), resulting in increased values of δ\(^{13}\)C compared to samples without extraction. However, several studies have reported unexpected changes in δ\(^{15}\)N values after lipid extraction (Sotiropoulos et al. 2004, Ricca et al. 2007, Logan et al. 2008), which is believed to be a consequence of the loss of proteins associated with lipid structures, such as lipid-membrane proteins (Sweeting et al. 2006). Therefore, testing the effects of lipid removal on both δ\(^{13}\)C and δ\(^{15}\)N values is highly relevant before applying SIA to address questions about diet and trophic ecology.

Due to the high correlation between the values of the carbon to nitrogen (C:N) ratio and the percentage of lipids, the C:N ratio itself has been used as an indicator of tissue lipid content for numerous tissues and organisms (e.g. Post et al. 2007). Following this, samples with C:N > 3.5 are considered to have a high lipid content (Post et al. 2007), making chemical extraction, or the use of normalization equations, necessary (Sweeting et al. 2006, Post et al. 2007). However, some studies found no support for the relationship between the bulk C:N ratio and the lipid percentage in organisms such as fish (Fagan et al. 2011), marine mammals (Wilson et al. 2014, Yurkowski et al. 2015), sea turtles (Medeiros et al. 2015), and invertebrates (Kiljunen et al. 2006). Therefore, the need for lipid extraction should be tested empirically before considering the tissue as free of lipid interference, even for tissues with C:N < 3.5 (Fagan et al. 2011, Yurkowski et al. 2015).

Studies involving various aspects of the biology and ecology of sea turtles have successfully applied SIA (Arthur et al. 2008, Lemons et al. 2011, Thomson et al. 2012, González Carman et al. 2014). In many of these studies, the lipid extraction of sea turtle tissues has been used as a standard protocol, often without testing the extraction efficiency (i.e. lipid removal) and interference on δ\(^{15}\)N values (Revelles et al. 2007, Cardona et al. 2009). On the other hand, other authors decided not to extract lipids mainly based on C:N < 3.5 values (Tomaszewicz et al. 2015, Prior et al. 2016).

Considering that the methodology used in preparing the samples can influence the results and, consequently, the interpretation of isotopic data, we analyzed the effect of lipid extraction on the isotopic values of C and N in skin and muscle of juvenile green turtles Chelonia mydas. We determined (1) whether the chemical removal of lipids from these tissues increases their δ\(^{13}\)C values, thus evidencing the presence of lipids in the sample, and (2) whether this procedure significantly affects the δ\(^{15}\)N values, either by increasing or decreasing them. The goal of the study was to assess the need for extracting lipids from skin and muscle samples of juvenile sea turtles.

**MATERIALS AND METHODS**

**Collection and sample preparation**

The samples of green turtles (n = 15) used in this study were from dead, stranded animals found along the southern coast of Rio Grande do Sul, Brazil (31°20’ S to 33°45’ S) between May 2013 and June 2014. The decomposition stage of the specimens was assessed and classified according to Duarte et al. (2011). All animals used were classified into Stages 1 or 2 (fresh carcass or initial decomposition, respectively). Stable isotope composition was considered not to be affected by these decomposition stages as evidenced by previous work (Payo-Payo et al. 2013). For each turtle, curved carapace length (CCL) was measured from the nuchal notch to the posterior-most tip of the carapace with a measuring tape (Bolten 1999). Individuals with CCL < 115 cm were considered to be juveniles according the mean size of nesting females from Brazil (Almeida et al. 2011). Samples of skin and muscle tissue of the right fore flipper (Revelles et al. 2007) were collected with for-
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caps and stainless steel razor blades, placed in labeled plastic bags, and then stored frozen at −20°C.

Paired skin and muscle samples were thawed, rinsed with distilled water, and dried in an oven at 60°C for 24 h. Each tissue sample (15 skin and 15 muscle) was divided into 2 parts and subjected to 2 treatments: with and without lipid extraction. Samples of the first group were extracted by a Soxhlet reflux using chloroform:methanol (2:1) for two 8 h cycles (Folch et al. 1957, Revelles et al. 2007). After the extraction, samples were washed with distilled water and dried in an oven at 60°C for 48 h. All samples (with and without extraction) were then ground to a fine powder with a mortar and pestle, weighed (between 0.5 and 0.9 mg) on a precision scale (AUY 220, Shimadzu), and stored in 5 × 9 mm tin capsules.

For the determination of the δ13C and δ15N ratios, all samples were sent to the Stable Isotope Core Laboratory at Washington State University (USA), and analyzed by a continuous flow isotope ratio mass spectrometer (Delta PlusXP, Thermo Finnigan). A conventional delta notation (δ) in parts per thousand (‰) was used to express the stable isotope ratios of the samples in relation to that of standards:

\[ \delta X = \left[ \frac{R_{\text{sample}}}{R_{\text{standard}}} - 1 \right] \times 1000 \]  

where \( X \) is 13C or 15N, and \( R \) denotes the heavier: lighter isotope ratio (13C:12C or 15N:14N) in the sample \( R_{\text{sample}} \) and standard \( R_{\text{standard}} \). The standard was Vienna Pee Dee Belemnite for δ13C values and atmospheric N2 for δ15N values. No replicates of the samples were analyzed; however, the analytical precision based on the standard deviations around the means for internal laboratory standards run at set intervals was high (≤0.1‰, both for δ13C and δ15N), thus results are still expected to be significant. The C:N ratio was calculated by dividing the %C by %N.

**Statistical analysis**

The assumptions of data normality and homoscedasticity were verified using the Shapiro-Wilk and Bartlett tests, respectively. To test whether there was a significant difference in the values of δ13C, δ15N, and the C:N ratio between samples of skin and muscle, and within lipid-extracted and control (i.e. without lipid extraction) samples within the same tissue, data were analyzed using paired t-tests, with a significance level of \( \alpha = 0.05 \). Simple linear regression models were used to test the relationship between Δδ13C (difference between δ13C values of control and lipid-extracted samples) and the C:N ratio of control samples for muscle and skin in order to test whether the C:N ratio of untreated samples could be an indicator of the need for lipid removal. All analyses were performed in the statistical environment R v. 3.1.3 (R Development Core Team 2013).

**RESULTS**

The CCL of the green turtles analyzed varied between 35 and 47 cm (mean ± SD: 39.8 ± 3.6 cm), thus all were considered juveniles. The δ13C values were significantly lower in muscle than in skin samples (paired t-tests, \( t = 0.14, p < 0.001 \) for lipid-extracted and control samples). Nitrogen isotope values, on the other side, did not differ between tissues (\( t = 0.14, p = 0.13 \) and \( t = 0.14, p = 0.14 \), for lipid-extracted and control samples, respectively). C:N ratios were significantly higher in muscle samples (\( t = 0.14, p < 0.001 \), for lipid-extracted and control samples). The values of δ13C in muscle were significantly lower in control samples than in lipid-extracted samples (\( t = 6.2, p < 0.0001 \)). Mean δ13C differences between control and lipid-extracted samples (Δδ13C) was 0.51‰. However, this difference was mainly driven by 3 samples that showed an increase of >0.5‰ after lipid extraction, with all other samples being less modified by the treatment (i.e. Δδ13C < 0.5‰; Fig. 1). Skin samples did not show differences between treatments (\( t = 0.88, p = 0.39 \), with a mean Δδ13C of 0.09‰ (Fig. 1, Table 1). Neither the regression between the C:N ratio of control samples and Δδ13C for muscle or for skin were significant (\( r^2 = 0.06, p = 0.38 \) and \( r^2 = 0.05, p = 0.40 \), for muscle and skin, respectively).

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**Fig. 1.** Relationship between Δδ13C (the difference between carbon isotope values) of control and lipid-extracted samples of skin (open circles) and muscle (black circles) of juvenile green turtles *Chelonia mydas* and the C:N ratio of control samples.
Lipid extraction had no significant effect on the average values of $\delta^{15}$N found for both tissues ($t = 1.7, p = 0.11$ and $t = 1.05, p = 0.31$ for muscle and skin, respectively; Table 1). The mean difference between $\delta^{15}$N values of control and lipid-extracted samples ($\Delta \delta^{15}$N) was 0.38 and 0.16‰ for muscle and skin, respectively. The extraction of lipids significantly modified the values of the C:N ratios of muscle samples ($t = 3.46, p < 0.05$) but not C:N ratios of skin ($t = 2.09, p = 0.05$; Table 1).

### Discussion

Our results show that the effect of lipid extraction (chloroform:methanol solution) on the $\delta^{13}$C values was significant when analyzing the muscle (increase in $\sim 0.5$‰) of juvenile *Chelonia mydas*, but did not affect the $\delta^{13}$C values of the skin. Further, significantly lower $\delta^{13}$C and higher C:N ratios were found in muscle samples in relation to those found in skin samples. This difference could be interpreted as evidence of a higher lipid content of muscle samples (Post et al. 2007). However, this difference was maintained when $\delta^{13}$C values in lipid-extracted samples were compared. Similar differences between these 2 tissues were also reported for loggerhead turtles *Caretta caretta* (Revelles et al. 2007) and for pinnipeds (Todd et al. 2010). Therefore, other factors such as the carbon isotope composition of individual amino acids within tissues (Martínez del Río et al. 2009, Fagan et al. 2011) may play a role in the differences found between $\delta^{13}$C values of both tissues.

We found a mean difference of $\sim 0.5$‰ in $\delta^{13}$C values of muscle, where some of the samples (see Fig. 1) showed increases in carbon isotope values higher than 0.5‰ after lipid extraction; thus, caution is required when deciding on the need for lipid extraction, especially when dealing with low sample sizes that may include such outliers. Moreover, Lesage et al. (2010) calculated the predicted error in prey contribution using Bayesian stable isotope mixing models and found that an error of 0.5‰ due to sample treatment of consumer tissues would introduce a considerable bias depending on the degree of distinctiveness of the end members of the mixing model. Thus, although small $\Delta \delta^{13}$C values were found for juvenile sea turtle muscle samples, we recommended testing for the effect of lipid extraction when using $\delta^{13}$C, before analyzing dietary ratios by Bayesian mixing models.

In the present study, although the mean C:N values of skin and muscle were lower than 3.5, lipid extraction significantly increased the $\delta^{13}$C values and lowered the C:N ratios of muscle. Similar results were found for bone tissue of loggerhead turtles, where although samples showed mean C:N ratios of 3.1, changes in $\delta^{13}$C values of $\sim 1.5$‰ were found after lipid extraction (Medeiros et al. 2015), thus concluding that the C:N ratio may not accurately predict the lipid content of samples. Likewise, the reason for the use of C:N as an indicator of the amount of lipids has been discussed in several other studies where tissues with different lipid proportions showed similar C:N ratios (Kiljnen et al. 2006, Fagan et al. 2011, Ruiz-Cooley et al. 2011, Ryan et al. 2012). This finding is also important, as most normalization models of $\delta^{13}$C values (e.g. McConnaughey & McRoy 1979, Post et al. 2007) rely on the C:N ratio as a proxy for the lipid content of the sample. Therefore, caution is required when applying these mathematical methods that use the C:N ratio as a model parameter for calculating lipid-free $\delta^{13}$C values.

$\delta^{15}$N values showed no significant differences after lipid extraction. This result was similar to that found for loggerhead sea turtle bone (Medeiros et al. 2015) and a variety of other aquatic consumers (Ingram et al. 2007, Ricca et al. 2007). However, in other studies, a change in $\delta^{15}$N values was observed (Bodin et al. 2007, Logan et al. 2008, Lesage et al. 2010, Ruiz-Cooley et al. 2011, Wilson et al. 2014) and was associated with the extraction of lipoprotein components that have low nitrogen and the binding of proteins with structural polar lipid components (Bodin et al. 2007, Ruiz-Cooley et al. 2011). Due to the inconsistency of lipid extraction effects on $\delta^{15}$N values, separate runs for carbon and nitrogen SIA are recommended (e.g. Post et al. 2007, Kojadinovic et al. 2009).

### Table 1. Carbon ($\delta^{13}$C) and nitrogen ($\delta^{15}$N) isotope values and C:N ratios of muscle and skin of juvenile green turtles *Chelonia mydas*. Mean ± SD, minimum, and maximum values for the control (without lipid extraction) and lipid-extracted samples are reported.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>$\delta^{13}$C (%)</th>
<th>$\delta^{15}$N (%)</th>
<th>C:N</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>With extraction</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Muscle</td>
<td>−17.2 ± 0.9</td>
<td>−18.4</td>
<td>−15.5</td>
</tr>
<tr>
<td>Skin</td>
<td>−15.2 ± 0.6</td>
<td>−16.2</td>
<td>−14.4</td>
</tr>
<tr>
<td><strong>Control</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Muscle</td>
<td>−15.3 ± 0.6</td>
<td>−16.1</td>
<td>−14.2</td>
</tr>
</tbody>
</table>
Both the low C:N ratios as well as the lack of lipid extraction effects on carbon isotope values of skin reported in this study showed that there is no need for lipid extraction of this tissue in young green turtles. However, this recommendation may not be valid for tissues from turtles that are in other life cycle phases. During the reproductive phase, the lipid content in adult females increases in the subcutaneous layers and organs to maintain metabolism during vitellogenesis (Hamann et al. 2002). Those authors observed that the concentration of triglycerides in the plasma is lower in non-breeding females and increases at the beginning of vitellogenesis and courting. Thus, the results presented here may not be applicable for SIA of the skin of sea turtles in other development phases, and testing the need for lipid extraction in tissues in these groups is recommended.

CONCLUSION

Given the observed changes in the δ\textsubscript{13}C values between control and lipid-extracted muscle samples that may be biologically significant, it is recommended that the lipid content of juvenile green turtle muscle should be accounted for through chemical lipid removal. On the other hand, the lipid extraction of skin samples seems not to be a necessary step in the case of juvenile green turtles, but this procedure needs to be tested in other life stages of this species. Finally, we advise caution when using the C:N ratio of muscle of juvenile green turtles in lipid correction models, as it may not accurately predict lipid-free δ\textsubscript{13}C values, at least for samples in the range of C:N ratios observed here (3.2 to 3.5). Future studies should include green turtles from other age classes/developmental stages in order to encompass a broad range of lipid contents in these tissues.

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2008), unfortunately doubling processing time and laboratory costs. However, our findings showed no undesired effects of lipid extraction on δ\textsubscript{15}N values when analyzing skin or muscle of young sea turtles using chloroform:methanol as the solvent.


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