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FEATURE ARTICLE

Effects of lipid extraction on the isotopic values of sea turtle bone collagen

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ABSTRACT: Many stable isotope analysis (SIA) studies aim to track protein, which is assimilated in animal tissues from their food sources, to assess feeding ecology, movements, and ontogenetic shifts of marine animals. Lipids are known to be a potential source of bias because they are depleted in ^{13}C compared to ^{12}C. Although lipids are usually removed before SIA, there is a lack of standardized analytical protocols for this procedure. We tested the effects of lipid extraction with 2 chemical solvents (chloroform-methanol and petroleum ether) in the humeri of the loggerhead sea turtle *Caretta caretta* to establish a standard protocol for processing the bone collagen of marine animals and to develop a mathematical correction. In both lipid extraction treatments, $\delta^{13}C$ values were higher than the control, but only lipid extraction with chloroform-methanol showed significant differences. By contrast, the $\delta^{15}N$ values were not affected by lipid extraction treatments with either solvent. The linear regression between the $C:N_{bulk}$ ratio and $\Delta^{13}C$ was not significant, which does not support the assumption that there is a predictable relationship between the C:N ratio and lipid content. Nevertheless, a significant positive relationship between $\Delta^{13}C$ and $\delta^{13}C_{bulk}$ was observed, but such a model is not recommended as a mathematical lipid correction method because the model efficiency had a negative value, which indicates that the mean value of $\delta^{13}C_{le}$ is a better predictor than the model itself. These results suggest that lipid extraction should be taken into account in SIA of bone collagen tissues for accurate $\delta^{13}C$ determination.

KEY WORDS: Collagen · Lipid extraction · Lipid normalization · Sea turtle · Reptiles · Stable isotope analysis

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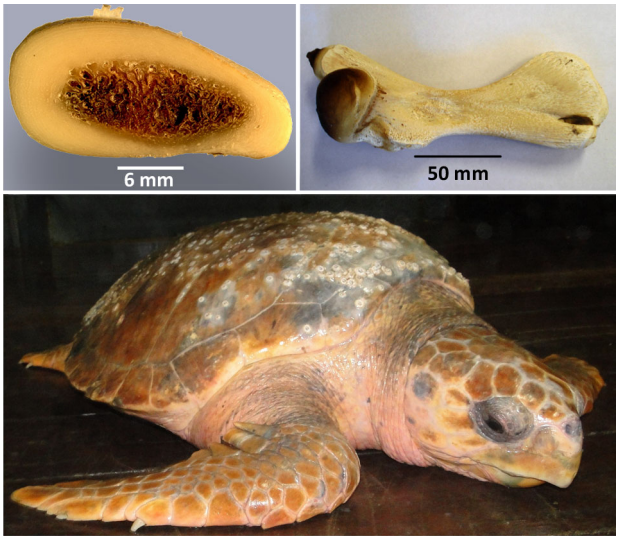

Whole humerus bone and cross section of loggerhead sea turtle *Caretta caretta* used for lipid extraction experiments

Photo: R. Petitet, L. Medeiros and NEMA archive

INTRODUCTION

Over the past several decades, stable isotope analysis (SIA) has become a useful tool in ecological research, particularly for identifying dietary sources and trophic relationships (e.g. Godley et al. 1998, Dodge et al. 2011), habitat use (Bjorndal & Bolten 2010, Pajuelo et al. 2012), migration patterns (Hobson 1999), and ontogenetic shifts (Arthur et al. 2008, Drago et al. 2009). SIA is a well-suited method for such studies because the stable isotope values of carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) reflect a time-integrated diet, i.e. the stable isotope ratio of con-

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sumer tissues reflects the similar ratio of its prey items within a timescale determined by the metabolic traits of each tissue and the species analyzed (Peterson & Fry 1987, Fry 2006). Due to preferential excretion of the lighter isotopes, and therefore the selection of heavier isotopes (Fry 2006), $\delta^{15}N$ values increase relatively predictably with each trophic level (DeNiro & Epstein 1981, Minagawa & Wada 1984) and can be used to assess the trophic level of a consumer. On the other hand, $\delta^{13}C$ is often used to discriminate food sources by using the large variation in the isotopic carbon value in food webs due to different photosynthetic baselines (Fry 2006).

An important methodological issue in SIA is that lipids have more negative $\delta^{13}C$ values relative to other biochemical compounds, such as proteins and carbohydrates (Gannes et al. 1997). The different nutrients in the diet are not used in the same way in the synthesis and maintenance of the different tissues of the consumer. Consequently, tissues often do not reflect the isotopic composition of the bulk diet, but instead represent the isotopic composition of the nutrient component of the diet from which the tissue was synthesized (Gannes et al. 1997). For animals that have high-protein diets, dietary proteins are exclusively used for the synthesis of tissues, whereas carbohydrates and lipids are catabolized to supply energy demands or stored in reserve tissues (Gannes et al. 1997). In lipid synthesis, kinetic effects that occur during the conversion of pyruvate to acetyl CoA cause a depletion in carbon stable isotope ratios of approximately 6 to 8‰ (DeNiro & Epstein 1977). Thus, the amount of lipid in bulk tissues could introduce a potential bias in $\delta^{13}C$ values for samples with higher lipid content and lead to misinterpretation of trophic relationships, particularly in studies using isotope mixing models (Kiljunen et al. 2006). Consequently, lipid-corrected $\delta^{13}C$ values more accurately reflect the carbon assimilated by consumers and provide more clear information about the magnitude of fractionation from prey to predators and relationships between them (D'Ambra et al. 2014).

According to Post et al. (2007), C:N > 3.5 in aquatic animals indicate the presence of lipids with the potential to alter $\delta^{13}C$ signatures; in such cases, lipid extraction or mathematical normalization is required. Questions have arisen regarding the general applicability of these thresholds and techniques (Fagan et al. 2011), and there is growing concern about the need to describe the effects on isotopic signatures caused by pre-analytical procedures, for instance sample acidification, distilled water rinsing, and lipid extraction (Mateo et al. 2008, Mintenbeck et al. 2008, Fagan et al. 2011). Recently, the usefulness of the C:N ratio as a predictor of lipid content was evaluated in fish tissues, and it was demonstrated that most mathematical normalization models utilized in SIA based on this parameter (e.g. McConnaughey & McRoy 1979, Post et al. 2007, Logan et al. 2008) underestimated the lipid content (Fagan et al. 2011). On the other hand, there is a body of evidence suggesting that lipid extraction procedures can remove nitrogen compounds (glycolipids or lipoproteins) and can lead to significant changes in $\delta^{15}N$ values (Post et al. 2007, Logan et al. 2008, Mateo et al. 2008). However, the standardization of sample processing protocols for isotopic analysis is still scarce for most tissues and taxa.

Given the differences in tissue composition, tissue selection is an important requirement for dietary reconstruction (Perkins et al. 2013). Bone collagen is largely composed of protein (Gannes et al. 1997) that turns over very slowly and has a long half-life, which for medium- and large-sized animals can reflect years or even decades (Dalerum & Angerbjörn 2005). Therefore, it is the most suitable tissue to identify long-term trends in animal dietary patterns, or changes in their habitat use, as well as historical changes (e.g. by comparison with museum specimens) (Dalerum & Angerbjörn 2005, Christensen & Richardson 2008, Drago et al. 2009). For example, a temporal change in stable isotope values was observed in bone collagen from North Sea harbor porpoises *Phocoena phocoena* in the period between 1848 and 2002. The recorded change in the isotopic values was associated with fisheries and provided a long-term temporal change in the structure of the pelagic ecosystem in the North Sea since the mid-20th century (Christensen & Richardson 2008). Moreover, SIA from the skull bones of South American sea lions *Otaria flavescens* from Chubut province (Argentina) in the scientific collection of the Centro Nacional Patagónico (CENPAT) was used to determine whether feeding habits changed during ontogeny (Drago et al. 2009). Results indicated that the contribution of benthic prey items to the diet of both sexes increases with developmental stage, except in senile males, and that first adults, i.e. sexually mature, but still growing in length, and adult males have a more benthic prey diet than females at the same developmental stage. Regarding $\delta^{15}N$ values, the trophic level of sea lions is roughly the same throughout their life, independent of the developmental stage and sex. Additionally, bone collagen is also useful in diet reconstructions and can provide evolutionary insights of dietary adaptations in pale-

ontology studies (Hobson & Schwarcz 1986). Thus, despite the fact that soft tissues (e.g. skin, blood, muscle, feathers) have been more frequently used for SIA of vertebrates, some questions could only be addressed by using tissues with a large time window, or that represent a long history of the individuals, such as bone collagen.

Recently, skeletochronology has been coupled with SIA to assess the ontogenetic shifts and early life stages of loggerhead sea turtles *Caretta caretta* (Snover et al. 2010, Avens et al. 2013). Skeletochronology is the study of growth marks in bones of reptiles and amphibians (Castanet & Smirina 1990), which allow researchers to estimate age and growth rate. Skeletochronology has been extensively applied over the last decade in studies with sea turtles (Avens & Snover 2013). Because bone tissues remain relatively inert after synthesis, the tissue retains the isotopic history of the animal, providing data on an annual scale, limited only by bone resorption at the core (Snover et al. 2010). For juvenile loggerhead sea turtles from North Carolina and Maryland (USA), a consistent growth mark pattern was identified by skeletochronology, and following SIA, clearly indicated that this growth mark pattern corresponded to a dietary shift from oceanic/pelagic to neritic/benthic stages (Snover et al. 2010). Based on the same approach, a recent study offers new insight into the juvenile oceanic stage of loggerhead sea turtles from the western North Atlantic. $\delta^{15}N$ values showed a significant relationship with growth rates, and mean oceanic duration was estimated at 12 to 13 yr (Avens et al. 2013). Although the combination of these 2 techniques is still in its infancy, it appears that this approach will greatly expand our knowledge on the complex life cycle of sea turtles.

Although SIA has the potential to make important contributions to animal ecology, the interpretation of the results relies on assumptions that are not well corroborated. For this reason, we tested the effects of lipid extraction with 2 common chemical solvents in the humerus bones of loggerhead sea turtles with the aim to establish a standard protocol for processing bone collagen from sea turtle species and to develop a mathematical normalization for SIA.

MATERIALS AND METHODS

Sample collection and processing

Samples consisted of 11 humeri from loggerhead turtles that were dead and stranded on the south coast of Rio Grande do Sul, southern Brazil, between Lagoa do Peixe (31° 20′ S, 51° 05′ W) and Arroio Chuí (33° 45′ S, 53° 22′ W); samples were collected from October to December 2009. For each turtle, curved carapace length (CCL) was measured from the nuchal notch to the posterior-most tip of the carapace with a flexible metric tape (±0.1 cm) (Bolten 1999). Humerus samples were macerated by immersing bones in fresh water for 2 to 3 wk until the total removal of soft tissues was achieved; bones were then air-dried for 2 wk. Three cross-sections (1 mm thick) were cut from each humerus with a low-speed isomet saw (Buehler®) with a diamond-embedded blade.

In order to assess the effects of lipid extraction on $\delta^{13}C$ and $\delta^{15}N$ values, we extracted lipids from the humerus sections of each individual turtle using 2 solutions: petroleum ether:ethyl ether (1:1) and chloroform:methanol (2:1) (hereafter referred to as petroleum ether and chloroform-methanol, respectively); samples were compared to a control with no lipid extracted. The removal of lipids for each group was performed with a Soxhlet apparatus for 4 h. All samples had carbonates removed by demineralization with 10% HCl using the 'drop-by-drop' technique (Jacob et al. 2005) until no gas bubbles were produced and were then washed with distilled water. The resultant collagen from the humerus subset samples was frozen at −20°C and then freeze-dried for at least 6 h. The assessment of lipid content among annuli was not the purpose of this study, but because humeri have a medullary cavity in the core filled with bone marrow lipids (Castanet & Smirina 1990), it is very plausible that lipid levels differ between the different growth marks, i.e., they may be higher in the center than at the edges.

The bone collagen of each section was powdered using a mortar and pestle, and ~1 mg of sample was placed in individual 4 × 6 mm tin cups and analyzed using a Thermo Finnigan Delta V isotope ratio mass spectrometer coupled to a Carlo Erba CHN, NA1500 Analyzer. The CHN Analyzer is coupled to the IRMS via the Thermo Finnigan Conflo III interface. Analyses were performed in the Laboratory of Analytical Chemistry, University of Georgia (USA). Stable isotope values are expressed in δ-notation as parts per thousand (‰) differences from the international standard material according to the following equation (as in Bond & Hobson 2012):

$$\delta X = (R_{sample} / R_{standard}) - 1 \quad (1)$$

where X is the ^{13}C or ^{15}N value, and R is the corresponding ratio of $^{13}C/^{12}C$ or $^{15}N/^{14}N$ (Peterson & Fry

1987). Vienna Pee Dee Belemnite limestone and atmospheric nitrogen (Air) were used as the carbon and nitrogen standards, respectively. Two internal laboratory standards (NIST 1570a and NIST 1577b) were analyzed for every 12 unknown samples. The measurement precision of both $\delta^{13}C$ and $\delta^{15}N$ analysis was 0.2‰.

Statistical analysis

Data were analyzed using a Shapiro-Wilk test to assess normality, and Bartlett's test was used to verify the homogeneity of variance between groups. One-way ANOVA was conducted separately for $\delta^{13}C$ and $\delta^{15}N$, followed by Tukey's pairwise post hoc comparisons when significant differences were found.

In order to evaluate the feasibility of mathematical normalization from our data set, simple linear regression models were performed to determine the relationship between (1) $\delta^{13}C$ values of lipid extracted ($\delta^{13}C_{le}$) and untreated samples ($\delta^{13}C_{bulk}$); (2) $\Delta^{13}C$ ($\delta^{13}C_{le} - \delta^{13}C_{bulk}$) and $C:N_{bulk}$; (3) $\Delta^{13}C$ and carbon content (%C); and (4) $\Delta^{13}C$ and $\delta^{13}C_{bulk}$. Model predictability was tested by performing model efficiency (EF) analysis as follows:

$$EF = 1 - \frac{\Sigma(y_i - x_i)^2}{\Sigma(y_i - \bar{y})^2} \quad (2)$$

where y_i is the $\delta^{13}C_{le}$ value, $\bar{y}$ is the mean of $\delta^{13}C_{le}$ values, and x_i is the model-predicted $\delta^{13}C$ value. EF values range between negative infinity and 1; values close to 1 indicate a 'near-perfect' model, whereas values close to 0 indicate a poor fit (Mayer & Butler 1993). Any model with negative values indicates that the mean value of $\delta^{13}C_{le}$ is a better predictor than the model itself. In our case, such a model would not be recommended as a mathematical lipid correction method. The significance level for all tests was $\alpha = 0.05$. All statistical analyses were carried out using R software version 3.0.3 (R Core Team 2014).

RESULTS

The CCL of the 11 sampled turtles ranged from 50.0 to 93.0 cm (mean ± SD: 73.8 ± 13.0 cm), representing juveniles and adults based on the size range of mature loggerhead sea turtles from Brazilian nesting areas in Espírito Santo state (83.0 to 120.0 cm CCL; Baptistotte et al. 2003) and Rio de Janeiro (86.5 to 114.5 cm; Lima et al. 2012). In both lipid extraction treatments, mean $\delta^{13}C$ values were higher than the control (Table 1). The values ranged from −16.96 to −13.36‰ (−15.00 ± 0.89‰) and from −15.37 to −13.10‰ (−14.38 ± 0.73‰) for the petroleum ether group and the chloroform-methanol group, respectively. The values for the control group ranged from −17.96 to −14.60‰ (−15.86 ± 1.08‰). One-way ANOVA comparing treatment groups showed differences in the $\delta^{13}C$ values ($F_{2,30} = 7.31$, $p < 0.05$), whereas Tukey's post hoc comparisons indicated that the $\delta^{13}C$ values were significantly higher after lipid extraction with chloroform-methanol relative to the non-extracted samples ($p < 0.05$). Non-significant differences were observed in values of the petroleum ether group relative to the control and the chloroform-methanol group. By contrast, the $\delta^{15}N$ values were not affected by the lipid extraction protocols ($F_{2,30} = 0.04$, $p = 0.95$). The mean $\Delta^{13}C$ was 0.86 ± 0.68‰ (range: 0.16–1.93‰) for the petroleum ether lipid extraction and 1.49 ± 1.07‰ (range: 0.03–2.99‰) for the chloroform-methanol treatment. The mean bulk C:N was 3.1 ± 0.6, with lower values after lipid extraction with the chloroform-methanol solvent and higher, but non-significant, values following the petroleum ether lipid extraction ($F_{2,30} = 2.10$, $p = 0.13$; Table 1).

Because the control and chloroform-methanol lipid extraction groups differed in the $\delta^{13}C$ values in bone collagen, the feasibility of mathematical normalization was tested using regression analysis. However, neither the relationship between $\delta^{13}C_{le}$ and the untreated samples nor between $C:N_{bulk}$ and $\Delta^{13}C$

Table 1. Stable carbon and nitrogen isotope values and C:N ratios in bone collagen of loggerhead sea turtles *Caretta caretta*. Samples were subjected to different lipid extraction treatments and compared to the control group. *Significant difference at $p < 0.05$ compared to control. Petroleum ether = petroleum ether:ethyl ether (1:1); chloroform-methanol = chloroform:methanol (2:1)

Treatment group	$\delta^{13}C$ (‰)			$\delta^{15}N$ (‰)			C:N		
	Mean ± SD	Min	Max	Mean ± SD	Min	Max	Mean ± SD	Min	Max
Petroleum ether	−15.00 ± 0.89	−16.96	−13.36	12.87 ± 1.83	10.4	15.41	3.6 ± 0.8	2.9	5.4
Chloroform-methanol	−14.38 ± 0.73*	−15.37	−13.10	12.99 ± 2.26	9.92	16.35	2.9 ± 0.8	1.0	4.7
Control	−15.86 ± 1.08	−17.96	−14.60	12.74 ± 2.06	10.17	16.56	3.1 ± 0.6	2.2	4.0

Table 2. Parameter estimates of linear regressions ($y = \beta x + \alpha$) for lipid normalization based on $\delta^{13}C_{bulk}$ or $C{:}N_{bulk}$ from a subset of samples of loggerhead sea turtle *Caretta caretta* bone collagen treated with chloroform-methanol vs. the control group; le: lipid extracted

Model	α	β	R^2	p
$\delta^{13}C_{le} = \beta.\delta^{13}C_{bulk} + \alpha$	−10.54	0.24	0.03	0.27
$\Delta^{13}C = \beta.\ C{:}N_{bulk} + \alpha$	0.002	0.47	−0.03	0.45
$\Delta^{13}C = \beta.\%C_{bulk} + \alpha$	0.76	0.01	−0.07	0.58
$\Delta^{13}C = \beta.\delta^{13}C_{bulk} + \alpha$	−10.54	−0.75	0.55	<0.01

were significant (Table 2). Hence, these regression results do not support the idea that there is a predictable relationship between $C{:}N_{bulk}$ and lipid content. The linear regression was also not significant for the $\Delta^{13}C$ and carbon content (%C) relationships. Nevertheless, a significant relationship was observed between $\Delta^{13}C$ and $\delta^{13}C_{bulk}$ ($R^2 = 0.55$, $p < 0.01$). Based on the linear regression coefficients of the latter model, we could potentially provide a lipid correction equation (Fig. 1). Nevertheless, despite the evidence of the relationship among $\Delta^{13}C$ and $\delta^{13}C_{bulk,}$ the model efficiency was negative (EF = −19 676), indicating that the model parameters should not be used as a lipid correction factor.

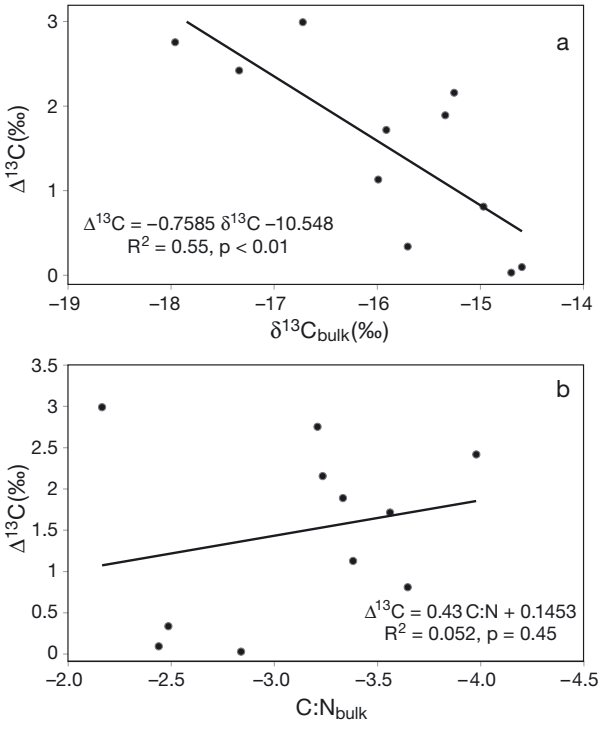

Fig. 1. Relationship between the change in carbon isotopic values ($\Delta^{13}C$) and (a) $\delta^{13}C_{bulk}$ and (b) the C:N ratio of bulk bone collagen of loggerhead sea turtles *Caretta caretta* before lipid extraction with the chloroform-methanol (2:1) solvent

DISCUSSION

To the best of our knowledge, this is the first study to measure the effects of lipid extraction methods on the isotopic values of bone collagen from loggerhead humeri. Because lipids have low $\delta^{13}C$ values compared with other compounds (DeNiro & Epstein 1977), it may confound the results of SIA and lead to erroneous ecological interpretations. The sea turtle humerus is a long bone that grows by collagen and calcium carbonate deposition, which is driven by physiological cycles synchronized with local environmental factors (Avens & Snover 2013). Although the use of bone tissue in SIA is still scarce, its recent application as a method of linking SIA with skeletochronology highlighted the usefulness of the analysis of this tissue to unravel the complex life cycle of sea turtles (Snover et al. 2010, Avens et al. 2013). The significant increase in $\delta^{13}C$ values observed after lipid removal using the chloroform-methanol solution suggests that lipid extraction is an important step for accurate $\delta^{13}C$ determination in sea turtle bone collagen. Therefore, lipid extraction should be taken into account in studies using the bone tissue of marine animals to assess their trophic ecology and ontogenetic shifts. Although studies on SIA of bones of sea turtles and seabirds are scarce (but see Silva et al. 2014), they are much more common in marine mammals, as bone is frequently the only material available from strandings and museum collections (Drago et al. 2009, Ciotti et al. 2014, Zenteno et al. 2015).

Overall, the $\delta^{13}C$ values in the bone tissue of loggerhead sea turtles decreased by approximately 1.5‰ after lipid extraction. The tissues of other species with high lipid contents showed similar changes in $\delta^{13}C$ values, such as in the muscle tissues of eel *Anguilla anguilla* and Baltic herring *Clupea harengus membras* (Kiljunen et al. 2006), the muscle and liver tissues of double-crested cormorant *Phalacrocorax auritus* (Doucette et al. 2010), and the skin of bottlenose dolphin *Tursiops truncatus* (Wilson et al. 2014). Although changes in $\delta^{13}C$ at this magnitude may appear biologically meaningless, it may influence the quantitative interpretation of food source partitioning when using mixing models or even in qualitative assumptions about movements and cryptic life stages. Kiljunen et al. (2006) demonstrated that the output of mixing models is largely influenced by whether the SI values of prey or consumer are lipid-normalized, and recommended caution in the interpretation of results from these models pending further experimental evidence.

The 2 different lipid extraction procedures, which used chemical solvents most commonly used in lipid extraction protocols, did not affect the $\delta^{15}N$ values of bone collagen. Similar to our results, a previous study showed no difference in the $\delta^{15}N$ values of skin of bottlenose dolphin following lipid extraction with chloroform-methanol (Wilson et al. 2014). Another experiment observed no lipid extraction effects in the nitrogen isotopic signatures of the exoskeleton and soft tissues of grain aphids *Sitobion avenae* (Perkins et al. 2013). By contrast, skin samples from 6 species of 4 families of cetaceans exhibited smaller, but statistically significant, changes in $\delta^{15}N$ values after lipid extraction, with a mean depletion of 0.14‰ in Balaenopteridae, and mean higher values of 0.30‰ and 0.22‰ in harbor porpoises and beluga *Delphinapterus leucas*, respectively (Lesage et al. 2010). For seabirds, the effects on nitrogen stable isotope values were observed in the muscle of white-tailed tropicbirds *Phaethon lepturus* in which the lipid-extracted $\delta^{15}N$ values were, on average, 0.20‰ higher than in bulk tissue (Kojadinovic et al. 2008).

Generally, the analysis of carbon and nitrogen isotopes in separate subsamples is recommended to ensure accuracy in $\delta^{15}N$ values (Sotiropoulos et al. 2004, Post et al. 2007, Kojadinovic et al. 2008). However, our data indicate that it is not necessary to separate subsamples of sea turtle bone collagen for SIA. This is a welcomed result because it represents a decrease in the cost of analysis and the amount of work required for processing samples in the laboratory. These results may apply to collagen samples collected from other vertebrates, particularly mammals and birds.

As an alternative to lipid extraction, models have been proposed for the mathematical normalization of $\delta^{13}C$ values (e.g. McConnaughey & McRoy 1979, Post et al. 2007, Logan et al. 2008). However, their applicability has been questioned because generalized corrections may be inaccurate for specific datasets (Fagan et al. 2011), and taxon-specific models have been proposed in some cases (Kiljunen et al. 2006, Sweeting et al. 2006, Bodin et al. 2007, Logan et al. 2008, Mintenbeck et al. 2008). Due to the high carbon content of lipids, mathematical normalization models are typically based on the correlation between tissue $C:N_{bulk}$ and the percent lipid content in the tissue (Post et al. 2007). This assumption is based on the premise that the lipid content contains negligible amounts of nitrogen and therefore the removal of any lipid amount is reflected in a decrease in the C:N ratio. Post et al. (2007) postulated that $C:N_{bulk}$ in tissues accurately predicts the $\Delta^{13}C$ among the lipid-extracted and untreated samples for a range of consumer species. For aquatic animals, C:N > 3.5 was established as a suitable indicator of the potential presence of a large fraction of lipids that could affect $\delta^{13}C$ analyses, and this predictor has since been widely applied (McClellan et al. 2010, Dodge et al. 2011, Pajuelo et al. 2012, Rosenblatt & Heithaus 2013). Although the utility of the C:N ratio as a predictor of lipid content was statistically validated (McConnaughey & McRoy 1979, Post et al. 2007, Logan et al. 2008), several studies noted that, due to species-specific variation in lipid composition, it could be difficult to apply a generic correction factor and recognized that any lipid-normalization method should be tested before application to a particular tissue (Kiljunen et al. 2006, Fagan et al. 2011, Ryan et al. 2012). For our dataset, it was not possible to employ the lipid normalization equation proposed by Post et al. (2007) to develop a new mathematical correction because the C:N ratio was not a suitable predictor of lipid content, and there was a weak correlation between $\Delta^{13}C$ and $\delta^{13}C$. We recognize that the limited sample size in our study could preclude the development of efficient models for lipid normalization, and more extensive studies are required to understand lipid content variation in humerus bones and the relationships associated with biochemical and physiological processes.

As expected, we observed a decrease in the C:N ratio following lipid extraction with chloroform-methanol. However, petroleum ether lipid extraction increased the C:N ratio. Chloroform-methanol is considered one of the most suitable chemicals for removing total lipid content, as it removes both polar and nonpolar lipids (Sotiropoulos et al. 2004). On the other hand, petroleum ether removes only polar lipids (Dobush et al. 1985). Thus, petroleum ether appears to be less effective, probably because it is unable to remove all the lipids in bone samples, which cause biases in the carbon and nitrogen stable isotope ratios. Our results are similar to those observed in muscle and liver of double-crested cormorants, in which the effects of lipid extraction were tested for chloroform-methanol and petroleum ether solvents (Doucette et al. 2010). Polar lipids were not removed with petroleum ether and consequently the lipid content was underestimated for both tissues. Therefore, the authors strongly recommend that lipid extraction be performed with chloroform-methanol. Nevertheless, there are many restrictions for chloroform use, for human and animal health reasons, and in many places the solvent is banned. In this case, if lipid extraction is con-

ducted with petroleum ether, $\delta^{13}C$ values must be adjusted (Doucette et al. 2010).

Another issue that has arisen in the methodological protocols of SIA regards the preservation methods. A study performed with blood and feathers of the spectacled petrel *Procellaria conspicillata* reported that the $\delta^{13}C$ values in ethanol showed high variation according to brand and batch and could account for the differences found in the $\delta^{13}C$ ratios in ethanol-preserved samples (Bugoni et al. 2008). Humeri do not require any chemical for preservation, but in our study, lipid extraction was performed with solvents that contain carbon, such as chloroform, methanol, and ether. It is possible that these chemicals may have influenced the carbon isotope values, similar to what was suggested for the spectacled petrel, and contributed to the variation in the C:N ratio in the present study.

Zug et al. (1986) validated that the humerus is the most suitable bone for age estimates of hard-shelled sea turtles. Because it is a long bone, its morphology is characterized by the presence of a medullary cavity filled by bone marrow (Castanet & Smirina 1990). Bone marrow varies considerably in composition, both within and between individuals, due to functional demand and age (Dietz 1946). For example, rabbit bone marrow in the center of the humerus is composed of water (54.8%), lipids (32.6%), lipid-free solids (12.6%), total nitrogen (1.94%), and lipid nitrogen (0.189%; Dietz 1946). The variation in bone marrow composition is a plausible explanation for the variability in stable isotope values between treatments, as well as the lack of a relationship between the C:N ratio and the $\Delta^{13}C$ or $\delta^{13}C$ values. It is also notable that bone maceration before processing for SIA could cause high variability in lipid content because maceration does not degrade marrow homogeneously, potentially causing an inherent lipid variation in bone tissue. Studies addressing the influence of maceration methods on the lipid content in bones are required and could improve our understanding on the observed variation in the stable isotopes of bone tissues.

CONCLUSION

In light of our findings, we strongly recommend that lipids be extracted from bone collagen of sea turtles, and probably other vertebrates, in studies involving SIA, in order to avoid biased ecological interpretations. Our findings show that lipid extraction is more effective when performed with 2:1 chloroform-methanol solvent and support that a single sample is sufficient for simultaneous determination of $\delta^{13}C$ and $\delta^{15}N$, because lipid extraction protocols did not affect $\delta^{15}N$ values.

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