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Effects of demineralization on the stable isotope analysis of bone samples

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RATIONALE: The sampling of sequential, annually formed bone growth layers for stable carbon ($\delta^{13}C$ values) and nitrogen ($\delta^{15}N$ values) isotope analysis (SIA) can provide a time series of foraging ecology data. To date, no standard protocol exists for the pre-SIA treatment of cortical samples taken from fresh, modern, bones.
METHODS: Based on the SIA of historical bone, it is assumed that fresh bone samples must be pre-treated with acid prior to SIA. Using an elemental analyzer coupled to an isotope ratio mass spectrometer to measure stable carbon and nitrogen ratios, we tested the need to acidify cortical bone powder with 0.25 M HCl prior to SIA to isolate bone collagen for the determination of $\delta^{13}C$ and $\delta^{15}N$ values. We also examined the need for lipid extraction to remove potential biases related to $\delta^{13}C$ analysis, based on a C:N ratio threshold of 3.5.
RESULTS: It was found that acidification of micromilled cortical bone samples from marine turtles does not affect their $\delta^{15}N$ values, and the small effect acidification has on $\delta^{13}C$ values can be mathematically corrected for, thus eliminating the need for pre-SIA acidification of cortical bone. The lipid content of the cortical bone samples was low, as measured by their C:N ratios, indicating that lipid extracting cortical bone samples from modern marine turtles is unnecessary.
CONCLUSIONS: We present a standard protocol for testing fresh, modern cortical bone samples prior to SIA, facilitating direct comparison of future studies. Based on the results obtained from marine turtle bones, pre-acidification and lipid removal of cortical bone are not recommended. This is especially useful as there is frequently not enough bone material removed via micromilling of sequential growth layers to accommodate both acid treatment and SIA. Copyright © 2015 John Wiley & Sons, Ltd.

Stable carbon ($\delta^{13}C$ values) and nitrogen ($\delta^{15}N$ values) isotope analysis (SIA) of animal tissues is used in ecology to estimate the diets and movement patterns of multiple taxa (e.g. Hobson *et al.*,[1] Newsome *et al.*[2]). The $\delta^{15}N$ values from animal tissues and other organic materials provide insight into trophic relationships, as they predictably increase with increasing trophic level.[3–6] Both $\delta^{15}N$ and $\delta^{13}C$ values can be used to estimate animal movement patterns as these values reflect the underlying nitrogen and carbon cycling processes that can be indicative of specific terrestrial or marine regions.[7–10] Findings from ecogeochemistry studies have addressed key ecological and life history questions critical to population conservation and management of multiple endangered and otherwise important species (e.g. Steller sea lions,[11] gray wolves,[12] humpback whales,[13] yellowfin tuna,[14] leatherback turtles,[15] African elephants,[16] and aquatic salamanders[17]).

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Stable isotope analysis (SIA) of multiple tissues from the same animal allows for the reconstruction of foraging and movement patterns at different time scales, as protein, and therefore isotopic, turnover varies depending upon the tissue sampled.[18–23] The hard, compact bone of the humerus, also called cortical bone, is formed during periosteal deposition and remains metabolically inert. As the humerus grows, outer compact layers are formed while the inner medullary cavity expands, resulting in the formation of new cortical bone and the resorption of some inner cortical layers. Once formed, cortical bone, a relatively inert tissue, does not have regular cellular turnover so SIA of cortical bone samples reflect an animal's diet during the time period from when that bone layer was produced. Sampling of an animal's whole bone can provide an integration of stable isotopes from multiple years, if not its entire lifetime. Alternatively, the inert layers of some structures such as teeth, or bones such as the humerus, are often formed sequentially, with each layer representing a different period of the animal's growth. Targeted sampling of these sequential inert layers allows SIA to provide a time series of isotopic data, reconstructing an animal's foraging ecology over time.[24–26]

Bone consists of two distinct components, collagen and bioapatite. Approximately one-third of bone by weight is collagen (~45% nitrogen and ~20% carbon, by collagen weight). Collagen is an organic matrix that makes up the protein component of bone and is assimilated from the carbon and nitrogen contained in the protein constituents of a consumer's diet.[27] The remaining two-thirds of bone by weight is composed of bioapatite [$Ca_{10}(PO_4)OH_2$]. An inorganic mineral, bioapatite can be ~3% carbon (typical range 2–7%) by weight when carbonate (CO_3^{2-}) substitutes for the phosphate (PO_4^{3-}) or hydroxyl (OH^-) group.[28–31] Bioapatite-based carbon is synthesized from a consumer's whole diet, which includes protein, carbohydrates, and lipids.[32,33] These two different bone components, collagen and bioapatite, have distinctive biosynthetic pathways. As a result, collagen and bioapatite experience differential isotopic fractionation and routing of dietary components during the biosynthesis of the bone tissue, and, when analyzed for their $\delta^{13}C$ and $\delta^{15}N$ values, reflect different portions of a consumer's diet.[27,34–37] For the best interpretation of an animal's foraging ecology from the stable carbon and nitrogen isotope ratios of bone, collagen is generally isolated from whole bone and targeted for SIA.[27,38] This allows for simultaneous sampling of carbon and nitrogen, while also avoiding the confusion of interpreting stable isotope ratio values from carbon bioapatite that are an amalgamation of stable carbon isotopes from multiple dietary components.[27,39–41]

For trophic studies utilizing SIA, collagen is typically isolated from whole bone with hydrochloric acid, and further processing beyond acidification is required for studies using whole bones and historical samples, to remove lipids, contaminants, and non-collagen protein remnants.[2,3,27,38,42,43] However, these treatments require large amounts of sample, much more than can be removed from an individual annual growth layer, and they subject bone tissue to extensive chemical processing that could affect stable isotope (SI) values, especially in fresh, modern samples, where contamination and digenesis are not expected. Medieros *et al.*[44] recently tested the effect of lipid extraction on whole cross sections of marine turtle bone, which included the vascularized, lipid-rich, central portion of bone where bone resorption and exchange of molecules with the blood occur. Our analyses are the first that we know of that focus solely on the need for acidification and lipid extraction on compact cortical bone. Our focus on cortical bone was for two reasons: (1) only the compact cortical bone contains the growth layers that allow for reconstruction of the sequential years of an animal's foraging ecology using SIA; and (2) the vascularized portion of bone, the medullary cavity, is different from cortical bone in its cellular structure and function, and therefore should be treated as a different type of bone tissue to avoid misinterpretation of bone SI values.[45] We developed standardized protocols to test for the effects of pre-SIA treatments on the SI values from bone growth layers to: (1) identify a simplified method to process cortical bone samples of modern, freshly collected bone, in order to obtain accurate, protein-based $\delta^{13}C$ and $\delta^{15}N$ values; and (2) estimate potential SI correction factors that can be used when limited amounts of bone material prevent pre-SIA treatment. Our simplified and standardized protocol can be applied to test any vertebrate bone with growth layers, will facilitate more efficient bone SIA studies, and will enable more direct comparison of results among future studies. Specifically, we tested the effectiveness of a simplified acidification method to isolate collagen from whole cortical bone by comparing the $\delta^{13}C$ and $\delta^{15}N$ values, and the percentage carbon (%C), and percentage nitrogen (%N) between treated (collagen) and untreated (whole bone) samples. We also assessed the need to lipid extract cortical bone samples. While tested specifically on marine turtle bones, these techniques may be applied to assess the need to treat, and define corrections for, other vertebrate bones.

EXPERIMENTAL

Marine turtle bone samples

We conducted experimental trials on cross sections of marine turtle humerus bones obtained following skeletochronology processes[46–48] to test for the need for lipid extraction and the effects of demineralizing acidification treatment on the accurate measurement of the $\delta^{13}C$ and $\delta^{15}N$ values from bone material. We also developed methods to best sample sequential growth layers for SIA from humerus bone cross sections collected from marine turtles and those data are presented elsewhere (Tomaszewicz *et al.*[26]). Humerus bones were collected from dead-stranded east Pacific green sea turtles (*Chelonia mydas*) (n = 15), North Pacific loggerhead sea turtles (*Caretta caretta*) (n = 15), and Northwest Atlantic loggerhead turtles (n = 20). Samples collected from the Pacific were from juvenile and sub-adult turtles of similar size at stranding (between 53 and 73 cm curved carapace length, CCL), whereas the Atlantic turtles were of similar size at stranding, but ranged from sub-adult to adult sizes (between 70 and 96 cm CCL). For Atlantic turtles, we converted any straight carapace length (SCL) measurements into curved carapace length (CCL) based on the SCL:CCL relationship presented in Snover *et al.*[24] (CCL = (SCL – 0.189) / 0.923, r^2 = 0.966). All Pacific turtles stranded between 2004 and 2011 at a 45-km stretch of beach, Playa San Lázaro, in Baja California Sur, Mexico, and the Atlantic turtles stranded between 1997 and 2011, primarily at beaches in North Carolina and Virginia, with three samples from Maryland and New Jersey.

Acidification of bone samples

We compared untreated bone samples with those that were treated with acid before SIA to isolate collagen by removing carbonate. For this study, we modified a method recommended by Chisholm *et al.*[42] and used in Godley *et al.*,[49] which uses hydrochloric acid (HCl), a weak acid, to isolate collagen. This method is similar to the preparation applied to the sampling of modern marine mammal teeth,[25] marine mammal cranial bone,[50] and chitonous and keratinized marine invertebrate tissues.[51–53] This method is notably simplified from the more complex protocols designed to prepare ancient and prehistoric bones for SIA which typically include steps for lipid extraction and the removal of decayed organic matter with sodium hydroxide.[3,36,41]

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To test the effect of pre-SIA acidification on bone, we created two, paired treatment groups (n = 50) from milled bone dust: (1) untreated bone and (2) bone treated with a weak HCl solution (Table 1). We cut the humerus bones to obtain 3-mm cross sections where the bone is most dense, just below the insertion scar,[45] using an Isomet slow-speed saw (Buehler, Lake Bluff, IL, USA) fitted with a diamond wafering blade (Buehler) (Fig. 1(a)). A diamond-studded dremel tool (RTX-3; Black and Decker, New Britain, CT, USA) was used to mill ~15 mg of powdered bone material from cross sections (Fig. 1). We took care to avoid the outermost edge of the bone and the inner vascularized portion of the bone, the medullary cavity, to prevent contamination, and sampled only the central portion of the cortical bone. We homogenized this powdered sample, and weighed and loaded 1.5 mg of untreated powder into a tin capsule for SIA. Of the remaining powder, 10 mg was placed into a 2-mL plastic, flat-bottomed vial for acidification.

We added 0.5 mL of 0.25 M HCl to the bone powder in the vial and stirred the contents with a flat spatula. Small amounts of CO_2 bubbles formed as the acid reacted with the inorganic carbon, and we stirred the acidic solution intermittently for 30 min at room temperature, then refrigerated it for 1–2 h. To ensure that the reaction was complete, the samples were stirred and, if no bubbles were observed, we centrifuged the contents for 2 min (5000 rpm, 2040 RCF centrifuge 5415C; Brinkmann Eppendorf, Hamburg, Germany). We pipetted off the HCl solution. The presence of a pale yellow to white precipitate was noted as an indication of pure collagen.[54] Precipitates with a brown coloration were also noted, as this could indicate possible contamination, impure collagen, or high lipid content.[54] The samples were rinsed with ultra-pure water three times by adding 0.5 mL of water, centrifuging the contents for 5 min (5000 rpm, 2040 RCF), then pipetting off the excess water. Following the third water rinse and centrifugation, we pipetted off only 0.25 mL of water, and the remaining water-collagen solution was mixed. We transferred this solution with a disposable plastic pipette directly into a pre-weighed tin capsule positioned in a 96-well plate. The samples were oven-dried at ~50°C for 48 h, then weighed and folded for SIA. The target sample weight was 0.6 mg for collagen. We analyzed three replicates each of 30 samples (10 Pacific, 20 Atlantic) to assess within-sample variation. The final analysis used single trial values, as no within-sample variation was observed (Supplementary Fig. S1 and Table S1, see Supporting Information). Paired samples (untreated and acidified) were then directly compared to assess the effects of acidification on the $\delta^{13}C$ and $\delta^{15}N$ values (Tables 1 and 2, and Supplementary Table S2, see Supporting Information).

Table 1. Experimental setup and design

		Micromilled powder	
Origin	Species	Turtle sample size	Samples with ×3 replicates
Pacific	*Chelonia mydas*	15	5
	Caretta caretta	15	5
Atlantic	*Caretta caretta*	20	20
Total sample size		50	30

Number of samples used for each experimental trial. Sample size is the number of sample pairs compared, and the number of samples with ×3 replicates used to assess within sample variation.

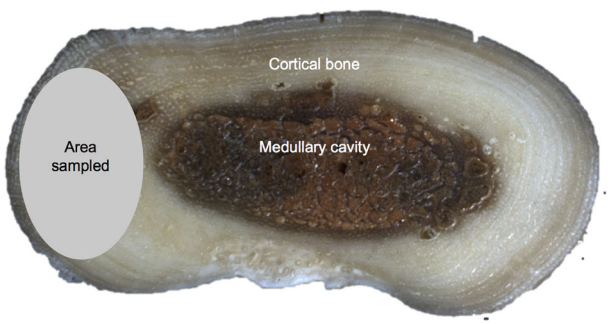

Figure 1. Green sea turtle (*Chelonia* mydas) humerus cross section, showing sampling location.

Stable isotope analysis

All the samples were analyzed for their $\delta^{13}C$ and $\delta^{15}N$ values, percentage carbon (%C), and percentage nitrogen (%N). The Pacific samples were analyzed by combustion in a Carlo Erba NA 1500 CNS elemental analyzer (Thermo Scientific, Milan, Italy) interfaced via a ConFlo II device (ThermoFinnigan, Bremen, Germany) to a Thermo Electron DeltaV Advantage isotope ratio mass spectrometer (Thermo Scientific, Bremen, Germany) in the Stable Isotope Geochemistry Lab at the University of Florida, Gainesville (Gainesville, FL, USA). Atlantic samples were similarly analyzed using a Carlo Erba NA 1500 CNS elemental analyzer interfaced with a DeltaPlusXL continuous-flow isotope ratio mass spectrometer (ThermoFinnigan) at the College of Earth, Ocean, and Atmospheric Sciences Stable Isotope Lab at Oregon State University, Corvallis (Corvallis, OR, USA). A conventional delta (δ) notation in parts per thousand or permil (‰) was used to express the stable isotope ratios of the samples relative to the isotope standards:

$$\delta X = \left(\left[R_{sample}/R_{standard}\right] - 1\right)$$

where the corresponding ratios of heavy to light isotopes ($^{13}C/^{12}C$ and $^{15}N/^{14}N$) in the sample and standard are represented by R_{sample} and $R_{standard}$, respectively. The standard for $\delta^{13}C$ values was Vienna Pee Dee Belemnite and for $\delta^{15}N$ values was atmospheric N_2. Laboratory reference materials, USGS40 (L-glutamic acid, $\delta^{15}N = -4.52$ ‰, $\delta^{13}C = -26.39$ ‰, supplied by the U.S. Geological Survey, Reston, VA, USA)) for Pacific samples, and caffeine (IAEA-600, $\delta^{15}N = +1.0$ ‰, $\delta^{13}C = -27.77$ ‰, supplied by the International Atomic Energy Agency, Vienna, Austria) for Atlantic samples, were calibrated at regular intervals against the standards. The precision for these data was determined using the standard deviations around the means for a subset of the internal laboratory standards run at set intervals. The standard deviation of the USGS40 data was 0.05 ‰ for $\delta^{13}C$ values and 0.08 ‰ for $\delta^{15}N$ values, and

Table 2. Acidification experiment effect

Bone ID	Species	Effect on $\delta^{13}C$ ($\delta^{13}C$ *untreated bone* – $\delta^{13}C$ *treated bone*)	Effect on $\delta^{15}N$ ($\delta^{15}N$ *untreated bone* – $\delta^{15}N$ *treated bone*)	CCL (cm)	Stranding year
Pacific					
F	Cc	−0.16	0.78	53	2011
G	Cc	−0.49	−0.13	59	2009
H	Cc	−0.36	0.66	73	2008
I	Cc	−0.35	0.35	65	2009
J	Cc	−0.34	0.06	69	2008
K	Cc	−1.37	−0.18	57	2008
L	Cc	−0.55	−0.38	53	2010
M	Cc	−0.13	−0.13	56	2011
N	Cc	−1.43	−0.47	64	2007
O	Cc	−1.53	0.28	51	2009
P	Cc	−0.62	−0.21	58	2009
Q	Cc	−0.63	0.17	63	2009
R	Cc	−0.35	0.13	69	2008
S	Cc	−0.75	−0.21	72	2008
T	Cc	−0.53	−0.31	72	2008
A	Cm	−0.09	0.22	53	2011
B	Cm	−0.30	0.69	56	2011
C	Cm	−0.26	0.56	73	2011
D	Cm	−0.08	−0.42	60	2009
E	Cm	−0.04	0.33	64	2010
U	Cm	−1.22	−0.56	55	2010
V	Cm	−0.91	−0.43	54	2011
W	Cm	−0.50	−0.56	65	2011
X	Cm	−0.67	−0.42	61	2011
Y	Cm	−0.61	0.39	53	2011
Z	Cm	−0.49	−0.16	62	2011
AA	Cm	−0.47	−0.10	59	2011
BB	Cm	−0.51	0.27	62	2004
CC	Cm	−0.37	0.06	52	2011
DD	Cm	−0.83	0.10	62	2011
AVERAGE:		−0.56	0.01	60.83	
	SD:	0.39	0.38	6.75	
Atlantic					
A	Cc	−0.84	−0.05	90	2007
B	Cc	−0.83	0.06	96	2008
C	Cc	−1.17	0.07	91	2004
D	Cc	−0.70	0.02	86	2005
E	Cc	−0.60	0.17	94	2005
F	Cc	−0.87	0.11	84	2005
G	Cc	−0.66	−0.28	87	2007
H	Cc	−1.18	−0.01	66	1997
I	Cc	−0.84	−0.05	94	2010
J	Cc	−0.87	0.13	72	1998
K	Cc	−0.72	0.10	92	2007
L	Cc	−0.75	0.11	94	2003
M	Cc	−1.07	−0.11	70	2003
N	Cc	−1.11	0.09	88	2007
O	Cc	−1.07	−0.29	85	1999
P	Cc	−0.61	0.10	89	2010
Q	Cc	−0.70	−0.15	66	1997
R	Cc	−0.90	−0.21	78	1998
S	Cc	−0.64	0.23	73	1997
T	Cc	−0.59	0.16	93	2011
AVERAGE:		−0.84	0.01	84.46	
	SD:	0.19	0.15	10.00	

Results of effect (difference) for both $\delta^{13}C$ and $\delta^{15}N$ values in permil (‰) units, from bone powder acidification experiment. The individual and mean (± SD) differences (e.g. $\delta^{15}N_{\text{untreated bone}} - \delta^{15}N_{\text{treated bone}}$ and similar for $\delta^{13}C$) in stable isotope values (‰) between acidified (treated) and non-acidified (untreated) bone powder from marine turtles, separated by ocean basin, Pacific and Atlantic. Turtle species are denoted as 'Cm' for *Chelonia mydas*, green turtles, and 'Cc' for *Caretta caretta*, loggerhead turtles.

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the standard deviation for the IAEA-600 data was 0.06 ‰ for $\delta^{13}C$ values and 0.07 ‰ for $\delta^{15}N$ values. The %C and %N were used to calculate C:N ratios (%C divided by %N) as well as to assess protein purity and material composition for the micromilled bone powder, based on established characteristic values.

Statistical analysis

We applied a two-sample, paired t-test on the stable isotope values from bone powder samples that were not treated (control) *vs* those that were treated with acid to evaluate the effects of acidification on the $\delta^{13}C$ and $\delta^{15}N$ values of milled bone powder samples. Similarly, a non-paired t-test compared the effects of acidification between samples from the two different ocean basins (Pacific *vs* Atlantic), and between different turtle species (green turtle *vs* loggerhead) in the Pacific. We applied a linear mixed-effects model with individual turtle as a random effect to examine variation attributed to treatment group and ocean basin of origin for the turtles (Pacific *vs* Atlantic). All the bone powder samples had C:N ratios of 3.5 or less. Therefore, in keeping with recommendations by Post[55] that samples from aquatic consumers with C:N ratios of 3.5 or less do not require lipid extraction, we included all samples in our analysis, resulting in a final paired sample size of n = 50 (Table 1). Finally, acidified milled bone powder samples from a total of 30 turtles (10 Pacific, 20 Atlantic) were run in triplicate to test for variation within the acidification treatment. The standard deviations between these triplicate samples were used to assess variation. We used the software program R for all analyses[56] and significance was tested at the $\alpha = 0.05$ level.

RESULTS

Effect of acidification

The acidification process was effective in isolating collagen from whole bone. The %C and %N from the acidified samples in this study were near those characterizing collagen (mean ± SD, %C: 43.01 ± 6.0 and %N: 14.26 ± 2.7; Supplementary Fig. S1 and Table S1, see Supporting Information). Whole bone, which is ~1/3 collagen and ~2/3 hydroxyapatite, is ~15% carbon and ~5% nitrogen and collagen is ~45% carbon and ~15% nitrogen by weight.[38] The C:N ratio of pure, unaltered protein, including collagen, ranges between 2.9 and 3.6,[38,54,57,58] and, while some of the untreated and acidified bone samples in the present study had C:N ratios less than 2.9, none were above 3.5 (Supplementary Fig. S2 and Table S1, see Supporting Information).

For the 30 bones that had acidified milled bone powder samples compared in triplicate, the overall sample replicate variation for the $\delta^{13}C$ and $\delta^{15}N$ values was very low (mean SD: ±0.08 ‰ and ±0.07 ‰, respectively). This observed variation was well below the measured precision for all stable isotope samples (<0.24 ‰ for both $\delta^{13}C$ and $\delta^{15}N$; Supplementary Fig. S1 and Table S1, see Supporting Information). Therefore, we randomly chose one each of the $\delta^{13}C$ and $\delta^{15}N$ values from the replicate samples for use in the analyses.

When samples from both species collected from the two ocean basins were analyzed together (n = 50 pairs), the $\delta^{13}C$ values were significantly different (paired t-test, $t = -13.5$, $df = 49$, $p < 0.01$) between acidified and untreated bone powder (mean ± SD: −0.67 ± 0.35‰; Supplementary Table S2, see Supporting Information). When examined by species, we found that the $\delta^{13}C$ values from acidified bone samples from green and loggerhead turtles collected in the Pacific were less than those from the untreated samples by 0.49 ± 0.33 ‰ and 0.65 ± 0.46 ‰, respectively. These species-specific differences were not significantly different from one another (unpaired t-test, $t = -1.03$, $df = 25.7$, $p = 0.31$), indicating that there was no species-specific effect of acidification on the bone $\delta^{13}C$ values from these two species. A basin-scale effect of acidification was detected, however. The $\delta^{13}C$ values from acidified Pacific samples (n = 30) and Atlantic samples (n = 20) were less than those from the untreated samples by −0.56 ± 0.39 ‰, and −0.84 ± 0.19 ‰, respectively, and these values were significantly different (unpaired t-test, $t = 3.2$, $df = 44.9$, $p < 0.01$). This suggests the potential for a differential effect of acidification depending on the location and/or size of the turtles. The Atlantic turtles in this study were larger (70–96 cm CCL *vs* 53–75 cm CCL for Pacific turtles; unpaired t-test, $t = -9.29$, $df = 30.6$, $p < 0.01$) and had higher average $\delta^{13}C$ values than the Pacific turtles (untreated means $\delta^{13}C$: Atlantic −14.3 ± 0.63 ‰ *vs* Pacific −16.0 ± 0.89 ‰; treated mean values $\delta^{13}C$: Atlantic −15.1 ± 0.70 ‰ *vs* Pacific −16.6 ± 1.10 ‰; Supplementary Table S2, see Supporting Information). The observed effects of acidification on all sample groups are three to four times greater than the maximum measured precision associated with the SIA processing (max. 0.24‰; Fig. 2(a)) and therefore may be biologically significant.

The effect of acidification treatment on $\delta^{13}C$ values for all bone samples was consistent within ocean basins. The $\delta^{13}C$ values from the treated and untreated Pacific samples were positively related to one another and were well described by a simple linear model (Fig. 2(a); treated Pacific bone samples = 1.2 × untreated bone samples + 2.1; $F_{1,28} = 230.4$, $p < 0.01$, adj. $R^2 = 0.89$), as were those from the Atlantic samples (Fig. 2(a); treated Atlantic bone samples = 1.1 × untreated bone samples + 0.2; $F_{1,18} = 232$, $p < 0.01$, adj. $R^2 = 0.92$).

There were no differences in the acidification effects on $\delta^{15}N$ for the bone powder from the two species of Pacific turtles analyzed (t-test, $t = 0.21$, $df = 27.7$, $p = 0.84$) or for turtles collected between the Pacific and Atlantic oceans (t-test, $t = 0.04$, $df = 40.6$, $p = 0.97$). As a result, all samples were analyzed together (n = 50) and the $\delta^{15}N$ values (mean ± SD) were not significantly different for the acidified (15.03 ± 2.03 ‰) *vs* the untreated (15.02 ± 2.05 ‰) samples, (paired t-test, $t = -0.27$, $df = 49$, $p = 0.79$; Supplementary Table S2, see Supporting Information).

As expected, there was a significant effect of acidification treatment on $\delta^{13}C$ values ($p < 0.01$), but no effect of acidification treatment on $\delta^{15}N$ values ($p = 0.97$) when we applied the linear mixed-effects model (Table 3). There was a significant effect of ocean basin ontest subsets of samples in order to asses the need for acidifi both $\delta^{13}C$ and $\delta^{15}N$ values ($p < 0.01$ for both), but the linear mixed-effects model showed no interaction effect of ocean basin and treatment for either $\delta^{13}C$ or $\delta^{15}N$ values ($p = 0.32$ and 0.99, respectively; Table 3).

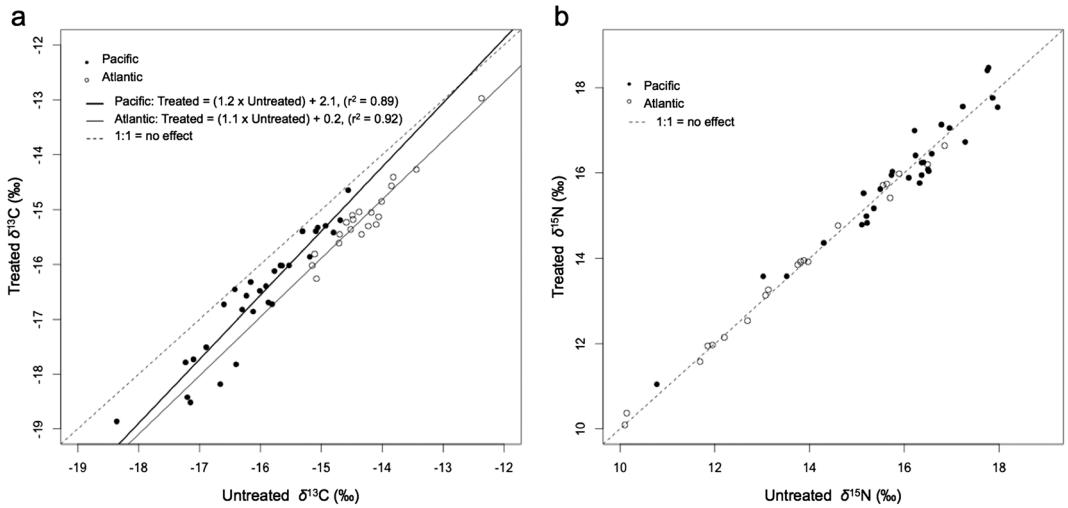

Figure 2. Effect of acidification treatment on micromilled bone powder on stable isotope values: (a) $\delta^{13}C$ and (b) $\delta^{15}N$ values.

Table 3. Linear mixed-effects model results. Test effect of ocean basin and acidification treatment on micromilled bone powder (Model: lme($\delta^{13}C$~Ocean*trt, random=~1 | Bone_ID. Similar model applied for $\delta^{15}N$).

Micromilled bone powder		Value	Std. Error	DF[a]	F-value[b]	p-value[c]
CARBON						
	(Intercept)	−15.10	0.19	67	13894.82	<.0001
	Ocean Basin	−1.49	0.20	67	127.75	<.0001
	Treatment (Acidification)	0.84	0.21	67	25.71	<.0001
	Interaction	−0.27	0.27	67	1.00	0.32
NITROGEN						
	(Intercept)	13.62	0.38	67	3817.38	<.0001
	Ocean Basin	2.32	0.41	67	61.73	<.0001
	Treatment (Acidification)	−0.01	0.43	67	0.00	0.97
	Interaction	0.00	0.56	67	0.00	1.00

[a] DF = degrees of freedom.
[b] F-value = F statistic from the linear mixed-effects model.
[c] p-value = significant at <0.05.

DISCUSSION

Stable isotope analysis

Effects of acidification on the $\delta^{13}C$ and $\delta^{15}N$ values

Acidification had a minimal effect on the $\delta^{13}C$ values, reducing Pacific and Atlantic samples by <1 ‰ (average ± SD 0.56 ± 0.39 ‰, and 0.84 ± 0.19 ‰, respectively). This effect of acid treatment on the bone powder samples was modeled by a simple linear regression equation, each with high adjusted R^2 values (Fig. 2(a)).

In situations where a difference of ~1 ‰ in $\delta^{13}C$ values may be biologically significant, application of the effect size as a constant offset (Pacific: −0.56 ‰; Atlantic −0.84 ‰) or the use of the equation (treated Pacific bone samples = 1.2 × untreated bone samples + 2.1; treated Atlantic bone samples = 1.1 × untreated bone samples + 0.2) will allow for the correction of the effect of acidification on the $\delta^{13}C$ values from untreated bone powder of modern turtle bones. This allows for comparisons of acid-treated bone samples that are representative of pure collagen and non-treated bone powder samples that are a mix of collagen and apatite. In addition, these correction options facilitate a simpler, quicker, and less costly analysis of bone powder samples; the need to acidify each sample to remove bioapatite-bound carbonate is eliminated.

To ensure that lipids were not affecting the $\delta^{13}C$ values from our bone samples, we used only samples with C:N ratios <3.5,[55] which included all samples in this study. Medeiros *et al.*[44] found an effect of lipid extraction on the $\delta^{13}C$ values from whole bone, but no effect on the bone $\delta^{15}N$ values. It should be noted that the primary difference between this and the current study is that fundamentally different tissues are being tested: whole bone in Medeiros *et al.*[44] *vs* cortical bone in the present study. Whole humerus cross sections include the central, vascularized,

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lipid-rich section of bone, whereas cortical bone is limited to bone powder extracted from outer growth layers. As a result, these two studies are not directly comparable.

Acidification did not affect the $\delta^{15}N$ values from the milled bone powder in the current study. This was expected because HCl should only react with bioapatite carbon, not with collagen-bound nitrogen. Therefore, the simplified acidification method, or no acidification treatment, is a viable option for pre-SIA treatment of bone when performing stable nitrogen isotope analysis of bone powder.

Mechanisms affecting $\delta^{13}C$ values

As expected with the isolation of collagen, the $\delta^{13}C$ values were lower when acidification eliminated the ^{13}C-enriched carbonate. The cause for this observed reduction in $\delta^{13}C$ values with acidification is caused by two factors: (1) the difference in the discrimination factors for the two bone components, collagen and bioapatite (see below); and (2) variation in the bioapatite content, and therefore the bioapatite-bound carbonate content, of cortical bone from marine turtles.

Consumer $\delta^{13}C$ values are generally higher than those from prey and the resulting difference is called a discrimination factor.[54,59] The carbon isotope discrimination factors are not known for either bioapatite or collagen from marine turtles, but these discrimination factors for other taxa (primarily mammals and birds) range from ~9 to ~14 ‰ and from ~3 to ~6 ‰, respectively, and are significantly larger than the commonly assumed 1 ‰ increase in $\delta^{13}C$ values.[27,34,36,37,60] As a result, the $\delta^{13}C$ values from collagen and bioapatite from the same individual would probably differ from one another by ~3 to 11 ‰. Yet in the present study, the difference between $\delta^{13}C$ values of collagen (acid treated bone) and whole (untreated) bone was very small (<1 ‰) for both Pacific and Atlantic samples. If the bioapatite content, and therefore carbonate content, of our marine turtle bones were significant, then, given that bones are ~2/3 bioapatite by weight and the difference expected in the $\delta^{13}C$ values from collagen and bioapatite could be ~3 to 11 ‰, one would expect the difference in the $\delta^{13}C$ values between these two bone components to be greater than the mean −0.67 ± 0.35‰ difference found in our study. Given this mean effect of less than 1‰, our results suggest that cortical bone from modern marine turtles contains very little bioapatite-bound carbonate. What small amount of bioapatite-bound carbonate is present, however, could account for the small difference in ^{13}C content between treated and untreated samples.

Carbonate content is the other key factor controlling whether or not the $\delta^{13}C$ values from bioapatite will contribute significantly to the $\delta^{13}C$ values from whole tissues.[52,61] Bone carbonate content varies, but averages ~3% by weight.[27–31] Recent studies using tissues with calcium-bound carbonate, but not specifically bone, have proposed using carbonate content, either measured directly or estimated by a 'carbonate proxy', to determine whether or not samples should be acidified prior to SIA.[52,53] There is agreement that tissues with low (3–9%) carbonate content (<5% in Serrano *et al.*,[53] <3–8% in Jacob *et al.*,[52] <9% in Mateo *et al.*[62]) do not warrant acidification treatment, as the effect of inorganic (i.e. bioapatite) ^{13}C would be insignificant and acidification treatments could unintentionally alter the $\delta^{13}C$ and $\delta^{15}N$ values from the tissue being sampled.[52,53,61,63] While these carbonate proxy studies did not test bones specifically, the carbonate content of bones is nonetheless an important factor for understanding how $\delta^{13}C$ values from inorganic carbonate might affect the $\delta^{13}C$ values of untreated bone samples. The contribution of carbonate to $\delta^{13}C$ values for modern marine turtle bones had not been previously tested, and confirmation of low carbonate content, and therefore effects on the $\delta^{13}C$ values, was necessary prior to using the bone $\delta^{13}C$ values to address ecological and biological questions about marine turtles. We note that measuring the actual carbonate content of cortical bone powder of marine turtles was beyond the scope of this study, and a future study focused on this would be valuable.

Our results suggest that the small differences in the $\delta^{13}C$ values between whole bone and collagen observed in this study (<1‰) are similar to results from other studies on samples with low carbonate content (reviewed in Schlacher and Connolly[63]). Therefore, we recommend that marine turtle cortical bone powder does not need acidification prior to SIA. Moreover, the $\delta^{13}C$ values should be corrected mathematically using the presented regression equation for the appropriate ocean basin (Pacific or Atlantic). Because carbonate content and other mechanisms affecting $\delta^{13}C$ values vary, the method presented here may be used in future bone SI studies to test subsets of samples in order to asses the need for acidification and/or lipid extraction, as well as determine regionally specific correction factors for different taxa.

Effect of turtle size, species, and ocean basin on $\delta^{13}C$ and $\delta^{15}N$ values

There were no differences in the effect of treatment on the $\delta^{13}C$ and $\delta^{15}N$ values between loggerhead and green turtles in the Pacific. There was a slight difference in the effect of treatment on the $\delta^{13}C$ values from turtle bone between ocean basins (Pacific *vs* Atlantic), but no difference in the $\delta^{15}N$ values. Nutrient cycling and oceanographic characteristic unique to each ocean basin prevent the direct comparison of actual SIA values.[10,64,65] However, we note that there were distinct size differences between the turtles from the two ocean basins with the Atlantic turtles being larger than the Pacific turtles, and the differences observed in the $\delta^{13}C$ values from turtles in the Pacific and Atlantic Oceans could be related to this size differential. Size is directly related to growth rate in marine turtles and has been shown to affect fractionation and resulting turtle tissue isotope values.[20,66] In addition, differences in diet between turtles in the two regions may affect the metabolic routing and fractionation of stable carbon isotopes and are another possible source of this observed variation.[67,68] Therefore, to test the effect of acidification treatment on the $\delta^{13}C$ and $\delta^{15}N$ values of modern cortical bone, we focused only on the observed difference between whole bone (untreated) and bone collagen (acidified) that we measured in marine turtle samples in each of the two ocean basins. Further exploration of the mechanism driving the observed difference of the effect of acidification on $\delta^{13}C$ values between samples from the two ocean basins was beyond the scope of this study, but warrants further investigation.

Despite potential sources of variation arising from ocean basin, species, or even turtle size, age, or sex, the high adjusted R^2 values from the linear models for $\delta^{13}C$ values from the Pacific and Atlantic turtle samples (R^2 = 0.89, and 0.92, respectively) support applying regionally specific mathematical offsets (constant value or linear equation) to correct the $\delta^{13}C$ values from unprocessed bone of marine turtles from the Pacific and Atlantic.

Sampling of bone for SIA and skeletochronology

Recent studies have attempted to combine SIA and skeletochronology on marine turtles (e.g. Snover *et al.*,[24] Avens *et al.*[69]), yet the lack of standardized protocols has limited the results. Only $\delta^{15}N$ values were measured in Avens *et al.*[69] due to the uncertainty of the $\delta^{13}C$ values that may result when collagen and bioapatite are analyzed together as whole bone. Snover *et al.*[24] presented $\delta^{13}C$ values for whole bone, yet no acid treatment was tested and lipids were extracted using dichloromethane. The standard protocol presented in the current study allows for the reliable use of $\delta^{13}C$ values, in addition to $\delta^{15}N$ values, through the application of a $\delta^{13}C$ correction value or the linear equation, and provides evidence to support eliminating lipid extraction for cortical bone. A standardized method for sequential sampling of annual bone growth layers is presented elsewhere[26] and, when used in conjunction with the recommended SIA processing and corrections in this paper, can produce a time series of SI patterns from individual animals.

RECOMMENDATIONS

The stable nitrogen isotope value of untreated whole bone reflects the $\delta^{15}N$ value of the dietary protein consumed at the time of bone synthesis, whereas the $\delta^{13}C$ value of untreated whole bone is slightly higher than that of the dietary protein alone due to the presence of small amounts of ^{13}C-enriched bioapatite-bound carbonate. Despite the small effect (<1‰) on $\delta^{13}C$ values, we present linear regression equations to mathematically correct for the $\delta^{13}C$ values from untreated bone samples from marine turtles collected in the Pacific and the Atlantic Oceans. Future studies on other vertebrates should employ these techniques to test a subset of samples to (1) determine if lipid extraction is necessary and (2) determine species- and location-specific correction regression equations. This is important for future studies, especially those utilizing samples from sequential growth layers where the amount of sample available is limited. To ensure that $\delta^{13}C$ values are not lower than expected due to the presence of lipid-bound carbon, we recommend analyzing only samples that fall at or below the C:N threshold of 3.5[55] to confirm that lipid extraction is not necessary. This is the first study to present a simplified standardized method to sample cortical, modern bone tissue for stable isotope analysis. These techniques, used to test and model the effect of acidification on marine turtle cortical bone, can be applied to a wide variety of both marine and terrestrial vertebrates, and the application of the standardized protocol presented here enables direct comparisons among future studies.

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