Methods for sampling sequential annual bone growth layers for stable isotope analysis

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Summary

1. Stable carbon (δ13C) and nitrogen (δ15N) isotope analysis (SIA) has proven useful in addressing fundamental questions in ecology such as reconstructing trophic interactions, habitat connections and climate regime shifts. The temporal scales over which SIA can be used to address ecological problems vary depending on the protein turnover times of the analysed tissue. Hard, inert tissues, such as teeth, bones and mollusc shells, grow in regular intervals (i.e. daily or annually), and sequential sampling of these growth layers provides a time series of isotopic patterns. As a result, SIA on these tissues is useful for elucidating behaviour and ecology of animals over time, especially those with cryptic life-history stages, such as marine turtles that retain growth layers in their humerus bones. To date, there exists no standard protocol for the sequential sampling of cortical bone samples taken from fresh, modern samples for SIA.

2. We tested two different methods, micromilling untreated bone cross sections and biopsy coring bone cross sections processed for skeletochronology, for sequentially sampling individual growth layers from marine turtle humerus bones.

3. We present a standard protocol for sequential bone growth layer sampling for SIA, facilitating direct comparison of future studies. We recommend using the micromilling sampling technique on untreated bone cross sections, as it facilitated higher precision sampling of growth layers that were not affected by chemical processing, and minimized sample handling, thereby reducing chances for contamination.

4. This is the first study to present a standardized method to sequentially sample annual bone growth layers for stable isotope analysis and facilitates direct comparison among future studies.

Key-words: bone, collagen, marine turtles, sequential sampling, skeletochronology, stable isotope analysis

Introduction

Stable carbon (δ13C) and nitrogen (δ15N) isotope analysis (SIA) of organic matter is a powerful tool used in ecological studies to elucidate diet, trophic level, habitat use and migration of a wide variety of taxa in both marine (e.g.Vander Zanden & Rasmussen 2001; Michener & Lajtha 2007; Graham et al. 2010; Newsome, Clementz & Koch 2010) and terrestrial (e.g. Koch, Fogel & Tuross 1994; Hobson, Barnett-Johnson & Cerling 2010) systems. Examination of both δ13C and δ15N values from animal tissues allows for reconstruction of animal movement patterns due to spatial variation in these values that reflect differential carbon and nitrogen processing at the base of terrestrial and marine food webs (DeNiro & Epstein 1978; Rau et al. 1983; Clementz & Koch 2001; McMahon, Hamady & Thorrold 2013).

Different tissues incorporate and retain stable isotopes from the diet at varying rates, allowing researchers to investigate foraging ecology over multiple time-scales by sampling-specific tissues (Hobson 1999; Dalerum & Angerbjörn 2005; Reich, Bjornal & Martinez del Rio 2008; Kurle 2009). Many hard tissues, such as bone, teeth, otoliths, corals and bivalve shells, do not have regular cellular turnover; instead, subsequent layers formed during growth are retained. These inert layers preserve their original chemical composition, thereby reflecting the stable isotope values of the environment and the prey consumed during the formation of a particular growth layer (e.g. Elorriaga-Verplancken et al. 2013). This creates a time series of data reflecting an animal’s diet and location when layers are formed at regular time intervals (e.g. days for otolith rings, or years for bone, tooth, coral and tree rings).

Sequential SIA of growth layers has been conducted on tissues such as otolith and teeth (e.g. Schwarz et al. 1998; Hobson 1999; Newsome et al. 2006; Elorriaga-Verplancken et al. 2013) with promising results for reconstructing habitat use patterns for migratory megafauna. For some marine turtle species, humerus bone tissue is deposited in annual layers (e.g. Snover et al. 2011) and, recently, sequential SIA of marine turtle bone growth layers identified by skeletochronology has been successful, generating a time series reflecting the diet and

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habitat of an individual turtle over multiple years (Snover et al. 2010; Avens et al. 2013). However, no standard sequential sampling methods have yet been described for SIA of $\delta^{13}$C and $\delta^{15}$N values from annual bone layers. Standardizing a protocol that combines these two methods, SIA with skeletochronology, could provide a reproducible approach to address important questions on the ecology and life history of many vertebrate species that do not possess teeth or otoliths, and could be especially useful for the study of migratory endangered animals such as marine turtles.

Here, we present and compare two methods to be used in combination with skeletochronology to sequentially sample individual bone growth layers for SIA. The establishment of a standard protocol will allow for future bone SIA studies to proceed with greater efficiency and accuracy, eliminate the potential for inconsistencies among methods examining ecological questions using bone SIA, and allow for more direct comparisons among studies. Our techniques were developed specifically for marine turtles, but can be applied to other species where annual growth in bone layers has been validated.

**Methods**

**MARINE TURTLE BONE SAMPLES**

We developed two methods, micromilling untreated bone cross sections and biopsy coring skeletochronology-processed bone cross sections, to sample sequential growth layers for SIA from marine turtle humerus bones obtained following sea turtle skeletochronology processes (Goshe et al. 2009; Avens et al. 2012). We also conducted experimental trials to test for the effects of inorganic carbon removed via acidification, and lipid extraction, on the accurate measure of the $\delta^{13}$C and $\delta^{15}$N values from bone material, and those data are presented elsewhere (Turner Tomaszewicz et al. 2015). Of relevance here, Turner Tomaszewicz et al. (2015) found that lipid content of cortical bone from modern turtles was low, based on the carbon:nitrogen ratio (C:N) of <3.5, thus negating the need for lipid extraction as recommended by Post et al. (2007). As part of a larger study, we collected the humerus bones from dead-stranded east Pacific green sea turtles (*Chelonia mydas*) ($n = 5$) and North Pacific loggerhead sea turtles (*Caretta caretta*) ($n = 5$). All samples collected were from juvenile and subadult turtles of similar size at stranding (between 53 and 73 cm curved carapace length, CCL), and all turtles stranded between 2004 and 2011 at a single beach (Playa San Lázaro) adjacent to a high-turtle density foraging area along the Pacific coast of Baja California Sur, Mexico (Seminoff et al. 2014).

**SEQUENTIAL SAMPLING OF BONE GROWTH LAYERS**

**Technique one: Micromilling**

Bones were prepared according to marine turtle skeletochronology processes as described in Goshe et al. 2009, 2010; and Avens et al. 2012; but modified for SIA sampling. Two 3-mm sections were cut from the whole bone using an Isomet slow-speed saw (Buehler) fitted with a diamond wafering blade (Buehler) (Fig. 1a). Next, the Isomet saw blade was used to make two 0.5-mm-deep notches in the dorsal side of both 3-mm sections, and these notches were used to align the two cross sections in later sequential sampling steps (Fig. 2b). After the notches were made, one cross section was chemically processed for skeletochronology and will be referred to as the ‘skeletochronology-

![Fig. 1. Experimental flow chart.](image-url)
processed’ cross section, whereas the second, paired cross section was not processed for skeletochronology, and hereafter referred to as the ‘untreated’ cross section (Fig. 1).

We identified individual growth layers in the bone sections with a skeletochronology-derived image that we call the ‘annual layer guide’. This guide is an image of the bone cross section showing each annual growth layer, which is separated by a distinct line of arrested growth (LAG). We labelled each LAG identified during the skeletochronology processing, and digitized the image (Snover & Hohn 2004; Goshe et al. 2009; Snover et al. 2011). After printing the annual layer guide image onto standard transparency film, the image was taped directly on a computer monitor. We then positioned the untreated bone section beneath an Olympus SZX10 microscope, fitted with an Olympus Spot-Flex camera (U-CMAD-2; Fig. 2a), and the image of the bone section was displayed on the computer monitor fitted with the annual layer guide transparency, and both images were aligned (Fig. 2a).

We used a computer-guided micromilling system (Carpenter Microsystems CM-2, version 3.0.6, Iowa City, IA, USA) for individual growth layer sampling. We programmed sampling paths using the CM-2 micromilling system and extracted ~1-5 mg of bone powder from individual growth layers of the untreated cross section, one growth layer at a time, using an NSK Volvere Vmax drill at 10 000 rpm, fitted with a 0.5-mm carbide, round-tipped bit (model RP 100, by Adhesive Systems Inc., Frankfort, IL, USA), and allowed to dry for at least 24 h prior to sampling. The 1.5 mg of bone dust from the sampling path of each growth layer was obtained by drilling to a depth of ~400 µm (10-µm increments over ~40 passes; Fig. 1b). To minimize the chance of sampling non-target growth layers, we avoided drilling deeper than ~400 µm because the location of LAGs often shifts slightly through the length of the bone, a common characteristic of growth layers. Upon completion of drilling each annual growth layer, we tapped the drilled bone powder onto a sheet of weigh paper, and weighed 1.5 mg of bone powder into tin capsules for SIA.

**Technique two: Biopsy coring**

Upon completion of skeletochronology chemical processing, a soft, flexible cross section is archived. We assessed the utility of sampling growth layers from these previously archived cross sections for SIA. We tested for this because, if these samples prove to successfully yield accurate δ13C and δ15N values, then a significant number of archived bone samples would become available for future SIA studies on marine turtle populations world-wide. The skeletochronology chemical processing leaves the cross sections pliable, precluding growth layer sampling with the micromilling technique and necessitating a different sequential sampling method we developed. Chemical processing steps and storage of these cross sections during skeletochronology include...
fixation for ~2 h in 10% formalin, followed by a water rinse, then
decalciﬁcation in a commercial agent that varies depending upon turtle
bone type (Snover & Hohn 2004; Goshe et al. 2009, 2010). This decalci-
ﬁcation step is analogous to the decalciﬁcation via acidiﬁcation that
was tested on cortical bone powder samples elsewhere (Turner Tomasz-
iewicz et al. 2015).

The skeletochronology-processed bone cross sections were soaked
for 6–37 h in RDO, a commercial decalciﬁcation agent consisting of HCl
(Apex Engineering, Aurora, IL, USA). Upon completion of the skele-
tochronology processing, the cross sections were archived in 100%
glycerine. All samples used in this study had been archived and stored
in glycerine for 1–2 years. Prior to use for sequential sampling in this
study, glycerine-archived samples were transferred to a 1:1 glycerine:
water solution for 1 day before transfer to soak in ultra-pure (MilliQ,
Darmstadt, Germany) water for 3 days. The water was changed daily.

Skeletochronology-processed cross sections were placed on, but not
affixed to, 25 × 75 mm glass slides, and positioned under the same
microscope and camera set-up used for the micromilling method. These
samples naturally adhered to the glass slides and were adjusted manu-
ally to align with the annual layer guide transparency affixed to the
computer monitor described above. Each skeletochronology-processed
cross section ranged from 1 to 3 mm in thickness, and we extracted
samples from each growth layer using 0.5-mm-diameter biopsy
punches (Harris Uni-core FTIR cardpunches, Ted Pella, Redding, CA).
This method is modiﬁed from one used to sample dentine growth rings for archaeological studies (Burt & Garvice-Lok 2013). The
biopsy punches removed small cores from the decalcified bone that
were ejected into a cryovial for further processing (Fig. 1c). In order to
obtain enough material for SIA, we removed a total of 4–10 cores from
each individual annual growth layer and, to accommodate the diameter
of the biopsy punch, we targeted annual layers that were at least
0.5 mm in width. In some cases, a scalpel was used to collect samples
from annual layers located near the outer edge of the cross section that
were too thin for proper removal with the biopsy punch. All biopsy
core samples were oven dried at ~50°C for 24–48 h before preparation
for SIA.

To ensure that complete demineralization occurred during the skele-
tochronology processing, a subset of growth layers (n = 26) were sam-
ples twice, and one sample of each pair was washed in with a weak acid
(0.25 M HCl), following the method described by Turner Tomaszewicz
et al. (2015; see Supporting Information for additional details). Paired
t-tests were used to examine the δ13C and δ15N values with and without
the HCl acid wash.

STABLE ISOTOPE ANALYSIS

We analysed all samples for δ13C, δ15N, per cent carbon (%C), and per
cent nitrogen (%N). Samples were analysed by combustion in a Carlo
Erba NA 1500 CNS elemental analyser interfaced via a ConFlow II
device to a Thermo Electron DeltaV Advantage isotope ratio mass
spectrometer in the Stable Isotope Geochemistry Laboratory at the
University of Florida, Gainesville. 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:

δX = ([Rsample / Rstandard] − 1) × 1000

where the corresponding ratios of heavy to light isotopes (13C/12C and
15N/14N) in the sample and standard are represented by Rsample and
Rstandard, respectively. Rstandard for δ13C was Vienna Pee Dee Belemnite
and Rstandard for δ15N was atmospheric N2. Laboratory reference mate-
rials, USGS40 (t-glutamic acid), were calibrated at regular intervals
against the standards. 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. Standard deviations for sam-
ples ranged from 0.03 to 0.20‰ for δ13C and from 0.02 to 0.24‰ for
δ15N, with mean (±SD) precisions of 0.08 ± 0.05‰ and 0.13 ± 0.08‰,
respectively.

The %N ratios for all samples were calculated by dividing %C by %
N, and we used the %C and %N values to assess protein purity and
material composition for the micromilled bone powder based on typi-
cal bone composition percentages. The %C and %N of whole bone is
generally ~15% and ~5%, respectively, whereas the %C and %N of
collagen is generally ~45% and ~15%, respectively. Pure, unaltered
protein, including collagen, has a C:N ratio between 2.9 and 3.6 (Schoe-
ninger et al. 1989; Ambrose 1990; Koch, Fogel & Turross 1994; Van
Klinken 1999).

STATISTICAL ANALYSIS

Absolute difference in δ13C and the δ15N values was compared between
the untreated micromilled samples and the skeletochronology-pro-
cessed biopsy core samples and was calculated as δ13Cskeletochronology-
processed – δ13Cuntreated, and similar for effect on δ15N. We evaluated
the effect of skeletochronology processing on the δ13C and δ15N values of
the biopsy cores using a linear mixed-effects model. Because this trial
included multiple samples from different years from individual turtles,
we assigned ‘year’ nested within ‘individual turtle’ as random factors to
examine variation attributed to skeletochronology treatment and turtle
species:

\[ \text{lm}(\delta^{13}\text{C} \text{ or } \delta^{15}\text{N}) \sim \text{Species*Treatment}, \text{ random} = ~ \sim 1/\text{Turtle/Year}. \]

Samples with low lipid content (C:N < 3.5 for aquatic consumers)
generally do not require lipid extraction for SIA (Post et al. 2007). We
eliminated any samples with C:N ratios >3:5. We used the software pro-
gram R for all analyses (R Core Development Team 2013), package
‘lme4’ for the linear mixed-effects model, and significance was tested at
the level of α = 0.05.

Results

The two sequential sampling methods tested, micromilling and
biopsy coring, were effective for physically extracting annual
bone growth layer samples. Samples from multiple growth lay-
ners were removed from each of the ten turtle bones. There was
no significant effect of the HCl wash on either δ13C values (t25 = −1.23, P = 0.23) or δ15N values (t25 = 0.03, P = 0.97)
of the skeletochronology-processed biopsy cores; therefore,
the rest of the analysis was conducted on the 60 biopsy core
samples that had been acidiﬁed. We extracted samples from a
total of 60 different annual growth layers from each of the
untreated cross sections, and the skeletochronology-processed
cross sections and directly compared these paired samples
(Table 1, Fig. 2c). The amount of time required to
extract a single annual growth layer sample by micromilling
was ~1–2 h, whereas a single sample (4–10 cores) removed by
biopsy coring took ~15 min.

The micromilling method more precisely sampled individual
growth layers, in comparison with the biopsy coring method, be-
cause the drill used to sample the untreated cross sections
remained in a fixed position and was constantly aligned with the annual image guide during sample extraction. In contrast, there was some unintentional and unavoidable movement of the hand-held biopsy punch during sampling of the skeletochronology-processed cross sections, resulting in possible misalignment with the annual image guide, and therefore lower confidence that every sample was removed from the intended growth layer. Further, the samples extracted using the micromilling method had a lower chance of contamination as they were only handled once, when the drilled bone powder was tapped on to a weigh sheet and then directly placed into a tin capsule for SIA. The biopsy core samples, contrastingly, were handled multiple times, potentially increasing the likelihood of a sample being contaminated. Repeated handling occurred (i) as each sample was initially collected, then oven dried, then weighed, and (ii) because multiple cores (4–10) were collected from each individual growth layer to collect enough mass for SIA.

WHOLE BONE POWDER VS. BONE CORES PROCESSED FOR SKELETOCHRONOLOGY

Based on the C:N ratios, %C and %N values, micromilled powder samples from the untreated cross sections reflected whole bone composition (%C 14.29 ± 2.48 and %N 4.41 ± 0.72), whereas the skeletochronology-processed biopsy core samples reflected characteristic collagen values (C% 42.87 ± 1.04 and %N 14.87 ± 0.38; Fig. S1, Table S1). The difference between the untreated micromilled samples and the skeletochronology-processed biopsy cores for δ¹³C was (mean ± SD) 0.16 ± 0.68‰ (range: −0.86 to 2.33 ‰) and 0.76 ± 0.99‰ for δ¹⁵N (range: −1.94 to 3.40 ‰; Table 2). The δ¹³C values were not significantly affected by the skeletochronology treatment based on results from the linear mixed-effects model (F₁,₂₈ = 3.14, P = 0.08). There was a slight effect of species on δ¹³C values (F₁,₂₈ = 5.39, P = 0.049), but there was no interaction between species and skeletochronology treatment (F₁,₂₈ = 0.22, P = 0.64; Fig. 3, Table 3). The linear mixed-effects model showed a significant effect of skeletochronology treatment on δ¹⁵N (F₁,₅₀ = 38.08, P < 0.0001). Species had no significant effect on δ¹⁵N values (F₁,₂₈ = 0.03, P = 0.88), yet there was an interaction between species and skeletochronology treatment (F₁,₂₈ = 6.48, P = 0.01; Fig. 3, Tables 2 and 3).

Discussion

COMPARISON OF TWO SEQUENTIAL SAMPLING METHODS

Sequential stable isotope sampling of bone growth layers can provide valuable information regarding animal diet and location over time. We showed that sequential samples from marine turtle bone could effectively be extracted from individual annual growth layers for SIA, thus creating a time series of stable isotope data for individual turtles. The ecological implications of the SIA results from some of the samples used in this study will be the focus of a larger, future study. Here, we presented two methods for sequential sampling of marine turtle bone growth layers, micromilling and biopsy coring, and determined micromilling to be the superior method.

The selection of micromilling as the best sequential sampling method was due to advantages in processing time and costs, ability to precisely sample thin annual layers, and consistency of stable isotope values. Although the cost of the biopsy core punches is low, this method still requires the same microscope equipped with a camera and computer used for the micromilling method. In addition, while the start-up cost of the micromilling software and drill is significant, the per-sample cost is reasonable given the durability and multiple applications of the equipment (e.g. sampling bones, teeth, otoliths and carapace scutes). Finally, while biopsy coring is a quicker process, the micromilling is automated once the sampling path has been programmed. Therefore, the amount of hands-on time required per micromilled sample is comparable to biopsy coring.

In addition, thin annual layers (0.10–0.25 mm width) can be sampled via micromilling, whereas fine sampling is often impossible with the biopsy coring method, even when a scalpel is used. The diameter of the biopsy tool itself (0.5 mm) limits the annual layers that can be sampled by the biopsy coring method. Further, micromill sampling of individual annual layers is more likely to be contained within the target growth layer, thus increasing sampling precision, whereas the larger biopsy core may inadvertently sample neighbouring growth layers. There is also less sample handling required for micromilling compared to biopsy coring, thus minimizing chances for contamination that could result from repeatedly handling samples.

Table 1. Experiment set-up and design

<table>
<thead>
<tr>
<th>Origin</th>
<th>Species</th>
<th>Turtle sample size</th>
<th>Micromilled Untreated</th>
<th>Skeletochronology-Processed w/HCl</th>
<th>Skeletochronology-Processed w/o HCl</th>
</tr>
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<tbody>
<tr>
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<td>Cm</td>
<td>5</td>
<td>27</td>
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<td>26</td>
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</table>

Number of samples used for the biopsy cores, all samples were from individual growth layers. The sample size for each group shown is the total number of samples from a unique turtle and year, for each species, that were compared as paired samples.

Table 2. Effect of skeletochronology processing on stable isotope values

<table>
<thead>
<tr>
<th>Bone ID</th>
<th>Annual growth layer year</th>
<th>Species</th>
<th>Effect on $\delta^{13}$C ($%_{\text{ov}}$)</th>
<th>Effect on $\delta^{15}$N ($%_{\text{ov}}$)</th>
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Table 2. (continued)

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Results of the difference between skeletochronology-processed samples and untreated samples for both $\delta^{13}$C and $\delta^{15}$N ($\delta^{15}$N_{skeletochronology} - $\delta^{15}$N_{untreated bone}, and similar for $\delta^{13}$C), in permil ($\%_{\text{ov}}$) units, from individual year growth layers. Values near 0.0 indicate similarity. Sample size represents number of sample pairs. See text for additional details.

Finally, the stable isotope values from the bone powder that was micromilled from the turtle growth layers were more predictable and consistent, and the mechanism driving any effects was understood (Turner Tomaszewicz et al. 2015). Samples that had been obtained via biopsy cores, however, were affected, likely by the skeletochronology processing, and the mechanism(s) causing these effects were unclear.

STABLE ISOTOPE ANALYSIS

The $\delta^{13}$C values from the skeletochronology-processed biopsy samples were not affected by the skeletochronology processing to a degree that surpassed the range of stable isotope measurement precision (mean difference was $+0.16 \pm 0.68 \%_{\text{ov}}$; maximum measurement precision for $\delta^{13}$C was 0.20\%_{\text{ov}}). Yet the maximum difference observed between these groups on $\delta^{13}$C values was $>3\%_{\text{ov}}$, which is greater than the variation observed for $\delta^{13}$C values from a recent study on the effects of acidification of bone cortical powder for stable isotope analysis (Turner Tomaszewicz et al. 2015). Further, the effect of skeletochronology processing on the $\delta^{13}$C values was not consistent or predictable among samples or $\delta^{13}$C values, thereby precluding the development of a useful correction value or equation. Also, the thorough water rinse was assumed to be sufficient for the removal of the storage solvent, glycerine, and a recent study on fish otoliths found no effect of storage in glycerine on $\delta^{13}$C (Gao et al. 2015), but the effect of storing bone samples in glycerine was not explicitly tested here. Finally, the mechanism(s) for the observed effects of skeletochronology processing, including glycerine storage, on the $\delta^{13}$C values from bone cores are unknown.

The $\delta^{15}$N values from the skeletochronology-processed biopsy core samples were affected (mean difference was $+0.76 \pm 0.99\%_{\text{ov}}$; maximum measurement precision for $\delta^{15}$N was 0.24\%_{\text{ov}}). We surmise that the effect of skeletochronology processing on the $\delta^{15}$N values was likely related to an unknown
alteration of the bone’s protein-bound nitrogen that occurs during the chemical processing required for skeletochronology. The mechanism(s) causing this alteration of \( \delta^{15}N \) values remain unknown. Other studies, including Turner Tomaszewicz et al. (2015), show that acidification should not affect the \( \delta^{15}N \) values of cortical bone powder samples. As a result of these potential effects of skeletochronology processing on both the \( \delta^{13}C \) and \( \delta^{15}N \) values, we do not recommended using these types of samples for future SIA when access to unprocessed bone is available.

**APPLICATION OF SEQUENTIAL ANNUAL BONE GROWTH LAYERS SAMPLING AND SKELETOCHRONOLOGY**

Sequential SIA of growth layers has been conducted on tissues such as otolith and teeth (e.g. Schwarcz et al. 1998; Hobson 1999; Newsome et al. 2006; McMahon et al. 2011; Elorriaga-Verplancken et al. 2013) with promising results for migratory megafauna. Only recently has sequential analysis of bone growth layers identified by skeletochronology been attempted (Snover et al. 2010; Avens et al. 2013), and until now, standard methods for sequential sampling for SIA of \( \delta^{13}C \) and \( \delta^{15}N \) values from annual bone layers have not previously been described. The standard protocol presented in the current study, together with the methods for SIA processing of bone in Turner Tomaszewicz et al. (2015), allows for the reliable use of \( \delta^{13}C \) and \( \delta^{15}N \) values from sequential bone growth layers.

In addition, sequential sampling and SIA of annual growth layers of marine turtle humerus bones provides a continuous, multiyear record of turtle habitat use that cannot be readily collected using traditional techniques. Satellite tag retention for marine turtles is typically on the scale of months to a year, and mark–recapture intervals are rarely annual. These methods gather data from a small fraction of the turtle’s life, while the sequential stable isotope sampling presented in this study collects information for multiple, sequential years of a turtle’s life, providing new and useful long-term information for marine turtle ecology and conservation.
Acknowledgements

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Data accessibility
All data used in this publication are provided within the text and in the affiliated Supporting information.

References


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**Supporting Information**

Additional Supporting Information may be found in the online version of this article.

**Appendix S1. Methods**

**Figure S1.** C:N ratios of biopsy cored skeletochronology samples.

**Table S1.** Biopsy core acidification experiment.