Title

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Journal

Rapid Communications in Mass Spectrometry, 38(19)

ISSN

0951-4198

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Publication Date

2024-10-15

DOI

10.1002/rcm.9874

Received: 22 March 2024 | Revised: 5 June 2024 | Accepted: 3 July 2024

DOI: 10.1002/rcm.9874

RESEARCH ARTICLE

Rapid Communications in Mass Spectrometry WILEY

Effects of pre-treatment, historical age, and sample characteristics on the stable isotope analyses of killer whale (*Orcinus orca*) bone

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Funding information
University of California, San Diego (UCSD); NIH Cell & Molecular Genetics Graduate Training Program; PADI Foundation; Lerner-Gray Fund for Marine Research by the American Museum of Natural History; Jeanne Marie Messier Memorial Endowed Fund (UCSD); American Cetacean Society Los Angeles Chapter John E. Heyning Research Grant; Association for Women in Science San Diego Chapter

Rationale: Stable isotope analysis of bone provides insight into animal foraging and allows for ecological reconstructions over time, however pre-treatment is required to isolate collagen. Pre-treatments typically consist of demineralization to remove inorganic components and/or lipid extraction to remove fats, but these protocols can differentially affect stable carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) isotope values depending on the chemicals, tissues, and/or species involved. Species-specific methodologies create a standard for comparability across studies and enhance understanding of collagen isolation from modern cetacean bone.

Methods: Elemental analyzers coupled to isotope ratio mass spectrometers were used to measure the $\delta^{13}C$ and $\delta^{15}N$ values of powdered killer whale (*Orcinus orca*) bone that was intact (control) or subjected to one of three experimental conditions: demineralized, lipid-extracted, and both demineralized and lipid-extracted. Additionally, C:N ratios were evaluated as a proxy for collagen purity. Lastly, correlations were examined between control C:N ratios vs. historical age and control C:N ratios vs. sample characteristics.

Results: No significant differences in the $\delta^{15}N$ values were observed for any of the experimental protocols. However, the $\delta^{13}C$ values were significantly increased by all three experimental protocols: demineralization, lipid extraction, and both treatments combined. The most influential protocol was both demineralization and lipid extraction. Measures of the C:N ratios were also significantly lowered by demineralization and both treatments combined, indicating the material was closer to pure collagen after the treatments. Collagen purity as indicated via C:N ratio was not correlated with historical age nor sample characteristics.

Conclusions: If only the $\delta^{15}N$ values from killer whale bone are of interest for analysis, no pre-treatment seems necessary. If the $\delta^{13}C$ values are of interest, samples should be both demineralized and lipid-extracted. As historical age and specimen characteristics are not correlated with sample contamination, all samples can be treated equally.

1 | INTRODUCTION

Analysis of the stable carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) isotope values in organismal tissues is an important tool in ecology for understanding animal habitat use, foraging habits, and trophic relationships.[1–5] In particular, stable isotope analysis of marine mammal tissues is increasingly prevalent[6] as this method can overcome the difficulties inherent to obtaining other types of foraging data from cryptic, at-sea species. One method by which to understand long-term temporal and spatial ecological patterns in the foraging and habitat use patterns of marine vertebrates involves serial sampling and stable isotope analysis of archived samples. This can be especially valuable for adding historical perspective to current patterns in the context of a rapidly changing oceanographic environment.

The stable isotope values measured from serially sampled material from accretionary hard structures such as teeth and bones provide particularly valuable data, as they enable insights into an animal's long-term (potentially lifelong) foraging patterns.[7] While teeth can offer annual resolution via distinct dentine layering, bones reflect an accumulation of foraging information over years[8,9] and can be collected from all marine mammal species even if they do not possess teeth. In addition, bone is a relatively accessible and consistent sample material from which to obtain biogeochemical data, as hard structures are more resistant to decomposition than soft tissues and bone remains have accumulated in natural history collections for centuries. Incorporating stable isotope data of bones from individuals across time and regions can collectively, and retroactively, build an ecological understanding of past baselines, current patterns, and future trajectories.

Bone comprises ~67% inorganic minerals (e.g., bioapatite) and ~33% organic protein, primarily type I collagen, by dry weight.[10] Importantly, the production of collagen is limited to dietary protein sources, whereas bioapatite is synthesized through a combination of dietary proteins, carbohydrates, and lipids.[11,12] Collagen is also less susceptible to environmental contamination (diagenesis) than bioapatite,[11–13] which readily absorbs external minerals and ions. To most accurately reflect the stable isotopes incorporated from the protein component of the diet, collagen must first be isolated from the inorganic bioapatite and non-target organic molecules, such as lipids and humic acids, which would introduce bias. Lipids are depleted in ^{13}C and have artificially lower measured $\delta^{13}C$ values in tissues,[1] and humic acids originate from exogenous soil and do not represent dietary sources of carbon.

There are three primary considerations for treatment of bone to isolate collagen[7,14]: removal of the inorganic bioapatite (called demineralization, acidification, or decalcification), removal of lipids (lipid extraction or delipidation), and removal of humic acids if the bones were buried or likely to be contaminated with decomposing organic soil matter. To verify that a treated sample has been reduced to pure collagen, one can measure the percentage carbon (%C), percentage nitrogen (%N), and the atomic carbon:nitrogen ratio ($C:N_{atomic}$),[14–16] the latter being the most commonly used metric. The widely accepted values are %C greater than 13, %N greater than 4.8, and a $C:N_{atomic}$ range of 2.9–3.6[7,16]. Note that Van Klinken[17] and Guiry and Szpak[14] have proposed a narrower range of acceptable $C:N_{atomic}$ ratios depending on collagen yield, taxa, and sample age.

Many different chemical methodologies have been utilized to isolate collagen, with varying effects on carbon and nitrogen content.[18–21] For example, hydrochloric acid (HCl), ethylenediaminetetraacetic acid (EDTA), and formic acid (CH_2O_2) have been used as demineralizing agents,[22,23] and chloroform-methanol and petroleum ether have been used to lipid-extract bone samples.[21] Sodium hydroxide and ultrafiltration have been used to treat archaeological marine mammal bones for humic acid contamination from soil,[24] although this is generally not a concern for modern marine mammal bones or bones that were collected before environmental burial and thus are not considered further in this study.

Previous research has shown inconsistent effects of demineralization, lipid extraction, and both protocols combined on the $\delta^{13}C$ and $\delta^{15}N$ values from teeth and bone depending on the chemicals, tissues, and species involved. For example, some studies have recommended against experimental demineralization after reporting no significant differences in the $\delta^{13}C$ and $\delta^{15}N$ values of untreated bone,[25] but others concluded experimental demineralization or a mathematical correction is necessary for accurate stable isotope measurementst.[22,26] Lipid extraction, again either experimentally or mathematically, is generally recommended for obtaining the $\delta^{13}C$ values from marine mammal bones that most accurately reflect those of their dietary proteins. This is because marine mammal bones have a high fat content and the $\delta^{13}C$ values of lipids are considerably lower than those of proteins, so need to be removed to avoid biased results.[27,28] Lipid extraction is not required if one is only interested in the $\delta^{15}N$ values from collagen.[25,26] Overall, there are few studies detailing the effects of collagen isolation specifically for modern marine animals, and the recommendations differ. Another complicating factor is the potential interaction between demineralization and lipid-extraction chemicals when used jointly.[25,29] As a result of the multiple chemical methodologies, different findings between taxa, and unpredictable isotopic interactions, it has been recommended that species-specific protocols be investigated for stable isotope analysis of bone collagen.[25,26,30]

In this study, we tested killer whale (*Orcinus orca*) bone samples under three treatment protocols alongside an untreated bone control: (1) demineralized with hydrochloric acid (HCl), (2) lipid-extracted with petroleum ether, and (3) both demineralized and lipid-extracted. Our first aim was to assess the effects of each treatment on the $\delta^{13}C$, $\delta^{15}N$, and $C:N_{atomic}$ values, and in doing so identify the treatment which most effectively isolates bone collagen for killer whales. Because our specimens were collected over the span of approximately 200 years, we were also able to investigate a second aim: whether there was a correlation between stable isotope values across the treatments and historical age of the specimen. We hypothesized that it may be more difficult to extract pure collagen due to degradation and contamination in specimens that were collected decades ago as opposed to recently. Lastly, we observed variation in individual bone powder characteristics (e.g., texture,

color), which we hypothesized could be related to contamination, possibly lipids or fungi, where samples with a grittier texture and darker color may have a higher contamination load. Our third aim was to identify if a correlation exists between control $C:N_{atomic}$ values as an indication of lipid or other contamination and bone powder characteristics. Our study provides a framework for any future stable isotope studies involving killer whale bone and contributes to the broader understanding of bone collagen isolation and stable isotope analysis.

2 | MATERIALS AND METHODS

We evaluated the $\delta^{13}C$, $\delta^{15}N$, and $C:N_{atomic}$ values from 34 killer whale bone specimens obtained from nine archived collections (Table S1) under a control condition and three experimental conditions used to isolate collagen and/or remove lipids from powdered bone. The killer whales lived in the North Pacific between the mid-late 1800s and 2016, with one exception that was collected on an unknown date. Some individuals had been captured and placed in captivity at some point in their life.

We took 31 bone samples from the skull: 28 from the occipital condoyle and three from dentary bone. We also took one bone sample each from a scapula, a hyoid, and a rib for a total of 34 killer whale bone samples. We preferentially chose skulls for sampling consistency since the head is often saved for museum collections when it is not feasible to salvage an entire skeleton. Furthermore, the back of the skull (e.g., the occipital condoyle) provides a discreet location for destructive sampling. For all skull samples and the scapula, we collected bone powder using a Dremel® tool (Racine, WI) at the lowest speed. We discarded the outermost shavings of bone to reduce contamination, and we rinsed and soaked the drill bit in 70% ethanol or isopropyl alcohol in between sampling. For the dentary, hyoid, and rib bones, we pulverized a small fragment in a manual coffee grinder which was sterilized with 10% bleach for at least 10 minutes, rinsed in water, and dried between specimens.

2.1 | Experimental treatments

We subjected bone samples to three experimental treatments alongside an untreated control: demineralization (D; HCl treatment), lipid extraction (LE; petroleum ether treatment), and both demineralization and lipid extraction (DLE). We extracted 65 mg of untreated bone powder from a specimen, then allocated 20 mg to each of the three treatments. We subsampled ~1.5 mg from the remaining 5 mg of untreated bone powder to use for the control condition.

For demineralization, we placed 20 mg of bone powder in 10 mL of 0.5 M HCl in a 15-mL glass tube. We covered the vials, left them for 48 h at room temperature in a fume hood, and then visually inspected the samples for evidence that the reaction had completed (e.g., a lack of bubbles that would indicate ongoing carbon dioxide release and the transition of the powder from opaque to translucent). All samples appeared to be fully decalcified within the allotted 48-h time period, at which point we centrifuged the samples and decanted the HCl. We rinsed each sample in ultra-pure water (MilliQ), centrifuged each again at 12 000*g*, and decanted the water five times before lyophilizing the remaining solid for 48 h. For the lipid-extraction treatment, we placed 20 mg of untreated bone powder into 15-mL glass tubes with 10 mL of petroleum ether. We placed the tubes in an ultrasonic bath set to 60°C and 40 KHz for 10 min, then centrifuged them at 12 000*g* for 5 min. We decanted the petroleum ether, and in all cases the supernatant was a very pale yellow, suggesting that the reaction had successfully extracted the lipids without need for further petroleum ether exposure. After decanting the petroleum ether, we added 10 mL of ultra-pure water to each tube for sonication under the same settings for another 10 min. Finally, we centrifuged the tubes for another 10 min, decanted the water, and dried the samples in either a drying oven at ~43°C or in a lyophilizer for 48 h. Although our drying method was constrained due to logistics, and this variable has not been explicitly tested for marine mammal bone at this time, note that Kaehler and Pakhomov[31] found no significant differences in the $\delta^{13}C$ or $\delta^{15}N$ values based on freeze- or oven-drying for tissues from marine vertebrate, invertebrate, and kelp species. For the combination treatment, we first demineralized and dried the allocated 20 mg of bone powder, then lipid-extracted and re-dried the sample as described above.

2.2 | Stable isotope analyses

We weighed ~1.5 mg of sample from each of the three treatments and the single control condition into 5 × 9-mm tin capsules for stable isotope analysis. Our samples were analyzed in two laboratories: the University of California, Santa Cruz (UCSC) Stable Isotope Laboratory using a CE Instruments NC2500 Elemental Analyzer coupled to a ThermoFisher Scientific DELTAplus XP isotope ratio mass spectrometer via a CONFLO III interface and the University of New Mexico (UNM) Center for Stable Isotopes using a Costech ECS 4010 Elemental Analyzer coupled to a ThermoFisher Scientific Delta V Advantage mass spectrometer via a CONFLO IV interface. All four conditions for 26 of the 34 killer whale bone samples were measured at UCSC, and all four conditions for the remaining eight samples were measured at UNM, meaning the relative changes in stable isotope values resulting from treatment were likely not due to variation between laboratories. The measured stable carbon and nitrogen isotope ratios are expressed in δ notation in parts per thousand (‰) relative to international standards, and the Vienna Peedee Belemnite (VPDB) for $\delta^{13}C$ and atmospheric nitrogen for $\delta^{15}N$ is $\delta^{13}C \text{ or } \delta^{15}N = [(R\text{sample} \div R\text{standard}) - 1] \times 1000$, where R is the ratio of ^{13}C:^{12}C or ^{15}N:^{14}N for the sample and standard.

Externally-calibrated standard reference materials across the laboratories (IAEA N1, IAEA N2, USGS 43, NBS 21, NBS 22, USGS 24, IU acetanilide, Pugel, and USGS 41) were measured intermittently for quality control and instrument precision. The overall standard

deviations from all reference materials were ≤0.1‰ for $\delta^{13}C$ values and ≤0.3‰ for $\delta^{15}N$ values. As $C:N_{atomic}$ should be used for the purposes of collagen quality control, $C:N_{weight}$ values were converted to $C:N_{atomic}$ values by multiplying by 12/14 where needed.[14] The results for the $\delta^{13}C$, $\delta^{15}N$, and $C:N_{atomic}$ values (hereafter C:N) are reported with subscripts reflecting each treatment: control (C), demineralization (D), lipid-extracted (LE), and both (DLE).

2.3 | Statistical analyses

We performed all statistical analyses with R v.4.2.1.[32] We used Sharpiro–Wilk and Mauchly's tests to examine the normality and sphericity assumptions, respectively, of a repeated measures analysis of variance (ANOVA). In the event of sphericity violation, the Greenhouse–Geisser correction was applied ('rstatix' package[33]).

The $\delta^{15}N$ values within all groups were distributed normally ($W = 0.96$ to 0.97, $p > 0.05$ for all). The $\delta^{13}C$ values from the lipid-extracted samples showed a minor violation of normality ($\delta^{13}C_{LE}$: $W = 0.89$, $p = 0.002$), but the other three treatment groups were consistent with a normal distribution ($W = 0.95$ to 0.99, $p > 0.05$ for all). We proceeded with the parametric repeated measures ANOVA, given the robustness to departures from normality.[34] The C:N values showed significant departures from normality for all groups ($W = 0.42$ to 0.80, $p < 0.001$ for all), so we applied a normal scores transformation using the Elfving method via the *blom* function of the 'rcompanion' package,[35] after which all group distributions except $C:N_{LE}$ ($W = 0.91$, $p = 0.013$) resembled normality ($W = 0.97$ to 0.99, $p > 0.05$ for all). Thus, we conducted repeated measures ANOVA tests for the $\delta^{13}C$, $\delta^{15}N$, and C:N values separately to investigate the effects of treatment on group means (e.g., means of the $\delta^{13}C_C$, $\delta^{13}C_D$, $\delta^{13}C_{LE}$, and $\delta^{13}C_{DLE}$ values were compared, and likewise for the $\delta^{15}N$ and C:N values). When the repeated measures ANOVA test detected a significant difference among group means at the $\alpha = 0.05$ level, we employed post hoc Tukey's honestly significant difference (HSD) tests with Bonferroni corrections to follow up with all pairwise comparisons and determine which group means were driving significance. We also used two-way repeated measures ANOVA tests to determine if there were interaction effects between the demineralization and lipid-extraction treatments for the $\delta^{13}C$, $\delta^{15}N$, and C:N values.[25]

To examine the relationship between the untransformed C:N (as a proxy for collagen purity) and the historical age of the specimen, we used the non-parametric Spearman's correlation coefficient for each treatment. We excluded six specimens from this analysis that did not have a precise collection date ($n = 28$). Lastly, we evaluated bone characteristics as a single parameter with three levels, given that texture and color were highly related: fine powder and white color, medium-coarse and cream color, or gritty and beige color. Each sample was assigned a state, which was converted to a contrast matrix, meaning the categorical assignments were converted to binary variables where 0 = not the assigned condition and 1 = the assigned condition for use in an ANOVA via linear regression. The linear regression model assessed control C:N values as a function of assigned bone characteristic state.

3 | RESULTS

We detected a global significant difference in the mean $\delta^{13}C$ values of 34 killer whale bones across the three treatments and the control condition ($F_{[1.99,\ 66)} = 11.30$, $p < 0.001$). The Tukey's HSD post hoc tests showed that the only non-significant comparison was between the $\delta^{13}C_D$ and $\delta^{13}C_{LE}$ values ($q = -0.93$, $p = 1.0$; see Table 1 for full output). Focusing on the three experimental treatments (D, LE, and DLE) compared to the control treatment (C), all three treatments resulted in significantly higher $\delta^{13}C$ values (Tukey's HSD test: $p < 0.05$) with reduced variation. The strongest relationship (Tukey's HSD: $q = 4.28$, $p < 0.001$) was between the $\delta^{13}C_C$ ($\bar{x} = -15.2 \pm 2.2$‰, means reported with SD hereafter) and $\delta^{13}C_{DLE}$ ($\bar{x} = -13.9 \pm 1.3$‰) values, meaning demineralization and lipid extraction combined resulted in the highest mean $\delta^{13}C$ value with the lowest variance among experimental treatments. The mean $\delta^{15}N$ values of killer whale bone were not significantly affected by any treatments ($F_{[1.65,\ 54)} = 1.13$, $p > 0.05$).

We also found that mean C:N values differed significantly ($F_{[2.33,\ 77)} = 41.36$, $p < 0.001$) among treatments. Focusing again on the comparisons of the three experimental treatments and the control treatment, post hoc analyses identified that demineralization ($C:N_C$ vs. $C:N_D$, $p < 0.001$) and the combination treatment ($C:N_C$ vs. $C:N_{DLE}$, $p < 0.001$) significantly lowered the C:N mean from the control value of 4.7 ± 1.7 to 4.0 ± 1.4 and 3.5 ± 0.4, respectively (Table 2; see Table 3 for full model output). It is particularly notable that the DLE treatment narrowed the C:N variation (SD) to 0.4, as compared to ~1.5 in all other treatments. The distributions of the $\delta^{13}C$, $\delta^{15}N$, and untransformed C:N values across treatments are illustrated in Figure 1 and summarized in Table 2. Overall, the $\delta^{13}C$ and C:N, but not $\delta^{15}N$, values were significantly affected by treatment, where the control condition had the lowest $\delta^{13}C$ and highest C:N mean values, and the DLE condition had the highest $\delta^{13}C$ and lowest C:N values.

We did not detect a significant interaction term between demineralization and lipid extraction for the $\delta^{13}C$ ($F_{(1,\ 33)} = 0.022$,

TABLE 1 Results of Tukey's HSD post hoc tests after a repeated measures ANOVA analyzing the $\delta^{13}C$ values of killer whale bone samples ($n = 34$) under three treatment conditions and a control.

Treatment 1	Treatment 2	Statistic	Adjusted *p* value
C	D	−3.076	0.025
C	DLE	−4.280	< 0.001
C	LE	−2.840	0.046
D	DLE	−3.314	0.013
D	LE	−0.930	1.00
DLE	LE	3.068	0.026

Abbreviations: C, control/untreated; D, demineralized; LE, lipid-extracted; DLE, both demineralized and lipid-extracted.

TABLE 2 The mean (±SD) $\delta^{13}C$, $\delta^{15}N$, and C:N values for killer whale bone samples ($n = 34$) across three treatments and a control are shown alongside the change in the $\delta^{13}C$ ($\Delta\ \delta^{13}C$) and $\delta^{15}N$ ($\Delta\delta^{15}N$) values for each treatment relative to the control.

Treatment	$\delta^{13}C$ (‰)	$\Delta\ \delta^{13}C$	$\delta^{15}N$ (‰)	$\Delta\ \delta^{15}N$	C:N	ΔC:N
C	−15.2 ± 2.2	–	18.0 ± 2.5	–	4.7 ± 1.7	–
D	−14.6 ± 1.9	0.6 ± 3.0	18.2 ± 2.3	0.1 ± 3.6	4.0 ± 1.4	0.7 ± 2.2
LE	−14.5 ± 1.8	0.7 ± 2.9	17.9 ± 2.5	0.1 ± 3.6	4.3 ± 1.5	0.4 ± 2.3
DLE	−13.9 ± 1.3	1.3 ± 2.6	18.1 ± 2.7	0.03 ± 3.7	3.5 ± 0.4	1.2 ± 1.8

Abbreviations: C, control/untreated; D, demineralized; LE, lipid-extracted; DLE, both demineralized and lipid-extracted.

TABLE 3 Results of Tukey's HSD post hoc tests after a repeated measures ANOVA analyzing the C:N values from killer whale bone samples ($n = 34$) under a normal score transformation using the Elfving method.

Treatment 1	Treatment 2	Statistic	Adjusted *p* value
C	D	6.647	< 0.001
C	DLE	8.284	< 0.001
C	LE	0.413	1.00
D	DLE	3.164	0.020
D	LE	−4.877	< 0.001
DLE	LE	−11.836	< 0.001

Abbreviations: C, control/untreated; D, demineralized; LE, lipid-extracted; DLE, both demineralized and lipid-extracted.

$p > 0.05$) or $\delta^{15}N$ ($F_{(1,\ 33)} = 0.032$, $p > 0.05$) values, but there was a significant interaction for C:N values ($F_{(1,\ 33)} = 11.630$, $p < 0.01$), albeit with a very small effect size ($\eta 2 = 0.0097$), meaning the interaction effect explains 0.97% of the variation in C:N values.

There was no significant correlation between the C:N values (as a collagen proxy) and the historical age of the specimens for any treatment (Spearman's correlation ρ for $C{:}N_C = 0.08$, $C{:}N_D = -0.16$, $C{:}N_{LE} = -0.08$, and $C{:}N_{DLE} = -0.21$; Figure 2). There was also no effect of bone characteristics on control C:N values ($F_{(2,\ 31)} = 0.688$, $p > 0.05$).

4 | DISCUSSION

4.1 | Effects of treatment on stable isotope values

Our primary aim was to investigate the effects of three treatments (demineralization, lipid extraction, and both demineralization and lipid extraction) relative to a control on the $\delta^{13}C$, $\delta^{15}N$, and C:N values of killer whale bone. Our results demonstrated that the $\delta^{15}N$ values from bone were not significantly affected by any of our treatments, suggesting that measurements of the $\delta^{15}N$ values from protein in bone are robust in the presence of lipids and inorganic molecules. The mean C:N value of control bone (4.7 ± 1.7) indicated that the untreated bone contained contaminated collagen (based on the pure collagen range of 2.9–3.6[7,16]), yet the mean $\delta^{15}N$ value of 18.0 ± 2.5‰ was nearly equal to the mean $\delta^{15}N$ value (18.1 ± 2.7‰) for collagen after receiving the DLE treatment. The DLE-treated collagen had a mean C:N value of 3.5 ± 0.4, which is more in alignment with that expected for "pure" collagen. Given this observation, untreated killer whale bone can likely be used for stable nitrogen isotope analysis, although we caution researchers to give attention to the C:N values from their analyzed bone samples and consider potential contaminating variables (e.g., fossilization, archaeological burial) that could influence stable nitrogen isotope values that were not represented in our relatively modern, museum-housed sample set. However, if the $\delta^{13}C$ values of killer whale bone are of interest, we recommend samples be both demineralized and lipid-extracted. This treatment (DLE) resulted in the lowest mean C:N value across treatments (3.5 ± 0.6) and the value closest to the acceptable range for pure collagen (2.9 to 3.6).[7,16]

The $\delta^{15}N$ values we found in our study align with other studies that suggest pre-treatment is not necessary for relatively modern marine animal bones.[25,26,36,37] However, Borrell *et al*.,[36] Pinela *et al*.,[37] and Tatsch *et al*.[25] all found no significant differences in the $\delta^{13}C$ values for bones left intact or treated with demineralization before stable isotope analysis for other cetacean species (*Balaenoptera physalus* or fin whale, *Delphinus* spp. or common dolphins, and Ziphiidae or beaked whales, respectively). Tatsch *et al*.[25] recommended lipid extraction before analysis of beaked whale $\delta^{13}C$ values, but cautioned against demineralization because of the presence of an interaction effect between both treatments on the $\delta^{13}C$ values. In contrast, we found that demineralization did have a significant effect on the $\delta^{13}C$ values from killer whale bones, and there was no interaction effect between demineralization and lipid extraction.

All three treatments led to significantly increased $\delta^{13}C$ values from killer whale bone, but the DLE treatment was associated with the most substantial change (Table 2). Stable carbon isotope ratios can be used to study animal movement and foraging dynamics,[38] and the change of 1.3 ± 2.6‰ we observed between the $\delta^{13}C_C$ and $\delta^{13}C_{DLE}$ values from killer whale bone is biologically significant given the variation in $\delta^{13}C$ values observed from different latitudes[39] and trophic levels.[39] As such, experimental treatments should be applied to the $\delta^{13}C$ values from untreated orca bone to avoid potential misinterpretations of killer whale foraging ecology.

The $\delta^{13}C$ values measured in the killer whale bone increased after lipid extraction and after demineralization, whereas the C:N ratios decreased. Lipid extraction and demineralization remove lipids and inorganic bioapatite, which is composed largely of lipids and carbohydrates (with some protein).[40] As lipids and carbohydrates

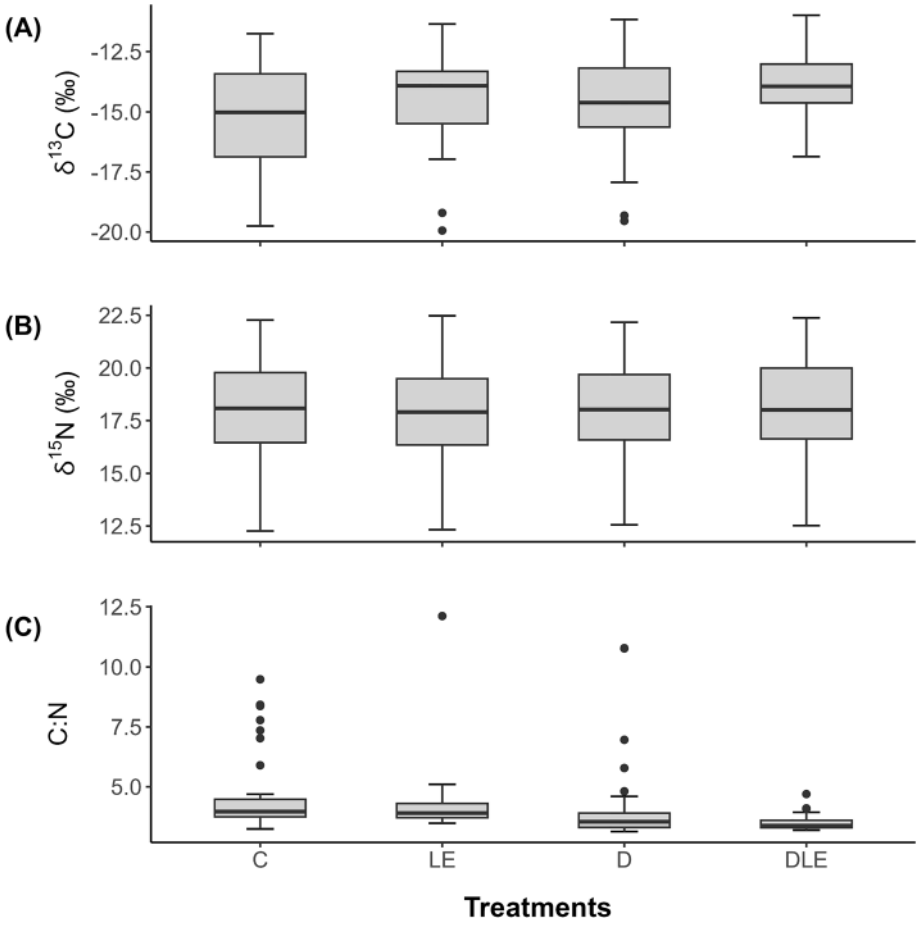

FIGURE 1 Boxplots of the (A) $\delta^{13}C$, (B), $\delta^{15}N$, and (C) C:N values from killer whale bone samples ($n = 34$) under an untreated control (C on the *x* axis) and three experimental conditions: lipid extracted (LE), demineralized (D), and both demineralized and lipid-extracted (DLE). The shaded boxes illustrate the interquartile range (the lower quartile, median, and upper quartile), and the whiskers extend to the minimum and maximum values. While there were no significant differences in the $\delta^{15}N$ values between treatments, the $\delta^{13}C$ values from the control samples did vary significantly from the means of all three experimental treatments. The C:N values also significantly differed with the D and DLE treatments, but not the LE treatment.

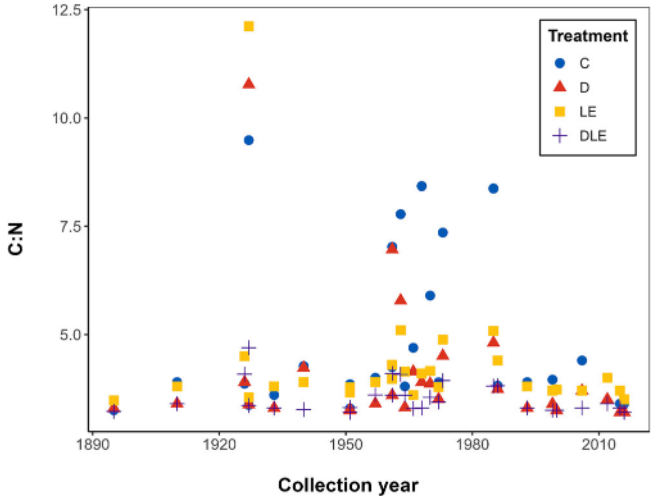

FIGURE 2 The C:N values from killer whale bones ($n = 28$) plotted against the year a specimen was collected. The treatment groups included the untreated control (C; blue circles), demineralized (D; red triangles), lipid-extracted (LE; yellow squares), and both demineralized and lipid-extracted (DLE; purple plus signs). [Color figure can be viewed at wileyonlinelibrary.com]

contain negligible nitrogen, but are partly structured by carbon (specifically the more abundant light isotope of carbon, ^{12}C),[41] it follows that the C:N ratios of treated bone would decline with the removal of ^{12}C, whereas the nitrogen content would remain relatively constant as removing carbon-based components would decrease their overall presence in the bone material while the nitrogen-based material would remain relatively unchanged. There is then a corresponding increase in the $\delta^{13}C$ (^{13}C:^{12}C) values as ^{12}C content is removed relative to ^{13}C, whereas the $\delta^{15}N$ values remain stable. Thus, it is likely the removal of the carbon-based lipids and bioapatite contributed to the changes in C:N ratios[42] we observed in killer whale bone across treatments.

Despite the small effect size, the significance of the interaction term between the demineralization and lipid-extraction treatments for C:N values in our study could also signal the importance of both treatments combined. In other words, the effect of a combined treatment is stronger than would be expected by the additive effect of each treatment alone. Our DLE protocol involved demineralization first and lipid extraction second, but other studies did the opposite.[21,25] Our reasoning was that the demineralization treatment might cause lipid in the bone to be more available for lipid extraction after demineralization, but future studies could test if the order in which a combined treatment occurs is important. We recommend both treatments be used for analysis of the $\delta^{13}C$ values from killer whale bones, especially when considering that the $\delta^{13}C$ values are sensitive to collagen contaminated with lipids and inorganic molecules[1] and C:N values indicating pure collagen were only reached for bone that had undergone both treatments. The physiological and

contextual (e.g., sample size, exact methodology) drivers of the discrepancies between species and studies are not yet clear, but the findings discussed here underscore the need to investigate patterns more widely and identify possible mechanisms for a priori predictions of treatment plans depending on taxa.

One source of potential variation in the $\delta^{13}C$, $\delta^{15}N$, and C:N values in response to the various treatments across multiple studies could be from the use of specific chemicals for each of the treatments. For example, we used petroleum ether for lipid extraction, and Tatsch *et al.*[25] used a chloroform-methanol solution. Stable isotope studies of bird muscle,[43] bird eggs,[44] and sea turtle bone[21] indicate that chloroform-methanol more effectively removes lipids (i.e., yields increased the $\delta^{13}C$ and decreased C:N values relative to a control) because it interacts with both polar and non-polar lipids,[45] whereas petroleum ether only removes polar lipids.[46] However, chloroform-methanol has also been shown to remove some non-lipids,[46] which may not be ideal for stable isotope analyses. In our study, lipid extraction via petroleum ether did have a significant effect on the $\delta^{13}C_C$, values, but it was the weakest effect ($p = 0.046$) among all significant comparisons; this could be due to the lower efficacy of petroleum ether as a solvent. Although chloroform-methanol may be more effective for lipid extraction, it is important to also consider toxicity and accessibility. Petroleum ether is a safer, widely permissible chemical with a simpler protocol, and in our case still significantly decreased the mean C:N values to approximately the acceptable collagen range (and increased the $\delta^{13}C$ values from the control treatment) when combined with demineralization.

With regard to isotopic variation from different demineralization protocols, Pang *et al.*[23] recommended either liquid solution HCl or EDTA over CH_2O_2 to most effectively remove inorganic material while preserving collagen integrity. Similarly, Wilson and Szpak[22] also found liquid HCl and EDTA performance to be comparable. These findings support our use of HCl as a demineralizing agent for killer whale bone. Although Tatsch *et al.*[25] also used HCl in their investigation of beaked whale bone, they utilized a vaporous form for 24 h, shorter than our 48-h exposure to liquid HCl. This procedural difference could have contributed to why we found a significant effect of demineralization on the $\delta^{13}C$ values of killer whale bone, but Tatsch *et al.*[25] did not find the same effect on beaked whale bone.

Another important consideration when applying various pre-treatments to bone specimens is the possibility of altering the $\delta^{13}C$ and $\delta^{15}N$ values of the collagen itself or influencing non-target molecules such that the tissue composition is undetectably changed without further chemical analyses. In our case, the aforementioned studies[22,23] supported the finding that HCl demineralization does not alter the $\delta^{13}C$ and $\delta^{15}N$ values of bone collagen. The consequences of lipid-extraction techniques are less clear, as some experimental conditions have shown altered $\delta^{15}N$ values as a result of lipid extraction.[47,48] Despite this, lipid extraction is still justified given evidence-based knowledge regarding the bias introduced from ^{13}C-depleted lipids[1] and ^{13}C-enriched bioapatite[49] relative to bone collagen. We were unable to conclusively determine that our petroleum ether protocol did not alter the $\delta^{13}C$ and $\delta^{15}N$ values from killer whale bone collagen or the chemical composition of our treated sample material. However, our data show the expected trend of an increase in the $\delta^{13}C$ values of the bone collagen when lipids were removed.

It appeared that some individual specimens in our study were somewhat resistant to decontamination treatments as their C:N values were never within the range of pure collagen. For example, specimen LACM 30461 showed the highest C:N value across all treatment groups, and even the DLE treatment only brought the C:N value down to 4.7 from 9.5 in the control treatment. Similarly, specimen USNM 594678 (also known as NMML 79) showed consistently elevated C:N values, ranking in the top five specimens with the highest C:N values for all treatments. Researchers may evaluate those unique cases to decide if exclusion from a study is appropriate. For cetaceans, there is precedence for an acceptable C:N upper limit as high as 4.0.[25,50–52] Higgs *et al.*[27] also suggested that the posterior end of cetacean skulls may have higher lipid content than the anterior and Tatsch *et al.*[25] speculated that a control C:N of 4.0 from the back skull represents relatively low lipid content. We primarily sampled from the occipital condoyle on the back of the skull, and our control killer whale C:N values aligned very closely with those of chloroform-methanol lipid-extracted beaked whales in Tatsch *et al.*,[25] with a mean around 3–4 and maximum of 8–9.

4.2 | Correlations with historical age and bone characteristics

We also investigated the potential for correlations between the C:N values from killer whale bones subjected to all treatments and the historical ages of the specimens. Although bone is typically highly resistant to degradation in a museum setting, older bones have had more time and opportunities to be exposed to fluctuating temperature, humidity, UV levels, pests, bacteria, and fungi, which can impact diagenesis[53–55] and lead to higher C:N values. Thus, we hypothesized that specimens collected decades in the past would be associated with higher C:N values (indicative of these age-related contaminations) than specimens collected in the 2000s. Our hypothesis was not supported for any of the treatments, meaning degradation and contamination were not significant factors when analyzing isotope values from bones housed for over approximately 100 years in museums.

We also observed variation in the bone characteristics of individual specimens, ranging from fine and white to gritty and beige. Texture and color could indicate varying levels of contamination; Huculak and Rogers[56] categorized a spectrum of bone colors resulting from humic acid exposure, fungal presence, and sun-bleaching. Bones with a high lipid load may also appear yellowish with a greasy texture, whereas bleached bone would be brittle. We categorized the killer whale bones in our study as fine powder and white, medium-coarse and cream, or gritty and beige. Although bright white specimens may have been either sun-bleached or intentionally bleached in the cleaning process, which degrades collagen,[57] we hypothesized that

fine white bone powder would exhibit $C:N_C$ values closer to the range of pure collagen than gritty, beige powder. We expected gritty, beige specimens were more likely to be contaminated by high lipid content or potentially fungal growth.[54,56] However, our hypothesis was not supported as we found no correlation between bone characteristics and the $C:N_C$ values. To further support that notion, we categorized specimen 594674 (also known as NMML 87) as gritty beige and documented on-site that the skull was especially greasy, yet the $C:N_C$ value (4.7) was equal to the mean (4.7) and this specimen responded well to the DLE treatment ($C:N_{DLE} = 3.3$). Tatsch *et al.*[25] mention that a high C:N value (between 8 and 9) in some individuals could be due to increased body fat or insufficient skull-cleaning procedures. Our results could indicate skull-cleaning procedures (or the environment prior to specimen collection) are more likely to cause C:N bias than fat content, as our top three highest $C:N_C$ values (9.5, 8.4, 8.4) were all from specimens categorized as fine white (i.e., possibly bleached), whereas a notably greasy specimen showed an average C:N value. However, additional studies examining the relationship between stable isotope values and sample preservation techniques are needed for more conclusive interpretations.

4.3 | Conclusions

Our results suggest that untreated samples may be used for the stable nitrogen isotope analysis of killer whale bone given relatively modern specimens housed in museums. However, we recommend both demineralization and lipid-extraction treatments for killer whale bone before determining its $\delta^{13}C$ values because both treatments change the $\delta^{13}C$ and C:N values for killer whale bone relative to control samples in ways that indicate the resulting material is closer to pure collagen (increased $\delta^{13}C$ and decreased C:N values) than samples left untreated. For concurrent analysis of the $\delta^{13}C$ and $\delta^{15}N$ values from killer whale bone, both measurements can be taken from a single sample that has been demineralized and lipid-extracted since the $\delta^{15}N$ values were not affected by the two treatments. Our recommendations for pre-treatment of marine mammal bones before stable carbon isotope analysis differ from those put forward by Tatsch *et al.*[25] for beaked whale bone, but we emphasize again that different chemical agents, treatment protocols, burial and preservation environments, etc. may influence these findings. Further studies investigating patterns among different taxa and physiological drivers of isotopic changes from chemical treatments are warranted. Additionally, we can recommend all killer whale bone specimens, regardless of historical age and bone characteristics, undergo the same treatments for stable isotope analysis, as these were not reliable indicators of potential contamination that could require treatment for removal.

AUTHOR CONTRIBUTIONS

Kelly R. Bowen: Conceptualization; data curation; formal analysis; funding acquisition; investigation; methodology; software; visualization; writing—original draft, review and editing. **Carolyn M. Kurle:** Conceptualization; funding acquisition; resources; supervision; writing—review and editing.

ACKNOWLEDGMENTS

We thank the natural history collections and all the staff/curators who made this work possible through specimen loans, including the American Museum of Natural History (N. Duncan, E. Hoeger), the Beaty Biodiversity Museum – University of British Columbia (M. deRoos, C. Stinson), the California Academy of Sciences (M. Flannery), the Natural History Museum of Los Angeles County (D. Janiger, J. Velez-Juarbe, S. Robson), the Museum of Vertebrate Zoology – University of California, Berkeley (C. Conroy), the NOAA Marine Mammal Laboratory (P. Clapham, P. Wade), the Museum of the North – University of Alaska (A. Gunderson, L. Olson), the Smithsonian National Museum of Natural History (M. Hawkins, D. Lunde, M. McGowen, J. Ososky), and the Burke Museum of Natural History and Culture – University of Washington (J. Bradley). Thanks also to D. Andreasen, C. Carney, and E. Cano at the University of California, Santa Cruz Stable Isotope Laboratory and S. Newsome and V. Atudorei at the University of New Mexico Center for Stable Isotopes for assistance with sample measurements. Additionally, we thank C. Turner Tomaszewicz for facilitating sample analyses and K. Lanigan for help with sample processing. We are also grateful for the time and feedback of two anonymous reviewers to improve this manuscript. Funding and support for K.B. and C.K. were provided by the University of California, San Diego (UCSD), the NIH Cell & Molecular Genetics Graduate Training Program, the PADI Foundation, the Lerner-Gray Fund for Marine Research by the American Museum of Natural History, the Jeanne Marie Messier Memorial Endowed Fund (UCSD), the American Cetacean Society Los Angeles Chapter John E. Heyning Research Grant, and the Association for Women in Science San Diego Chapter.

PEER REVIEW

The peer review history for this article is available at https://www.webofscience.com/api/gateway/wos/peer-review/10.1002/rcm.9874.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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How to cite this article: Bowen KR, Kurle CM. Effects of pre-treatment, historical age, and sample characteristics on the stable isotope analyses of killer whale (*Orcinus orca*) bone. *Rapid Commun Mass Spectrom*. 2024;38(19):e9874. doi:10.1002/rcm.9874