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### Authors

Bowen, Kelly

Kurle, Carolyn



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# Testing Assumptions for Stable Isotope Analysis of Marine Mammal Dentin Growth Layer Groups

Kelly R. Bowen | Carolyn M. Kurle

School of Biological Sciences, Department of Ecology, Behavior, and Evolution, University of California, San Diego, La Jolla, California, USA

**Correspondence:** Kelly R. Bowen (flandersrae@gmail.com)

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## ABSTRACT

**Rationale:** Stable isotope analysis of growth layer groups (GLGs) in mammal dentin is an increasingly prevalent and noninvasive approach to study animal foraging ecology. However, empirical evidence to support assumed proper methodologies for sampling GLGs is lacking. Here, we examine the effects of intratooth and intertooth variations with respect to targeted GLGs, as well as the effects of common pretreatments (e.g., formic acid and graphite) to enhance GLG visibility, on stable isotope values ($\delta^{13}C$ and $\delta^{15}N$) from dentin.

**Methods:** We measured the $\delta^{13}C$ and $\delta^{15}N$ values of killer whale (*Orcinus orca*) dentin. We used dentin from 37 teeth to compare stable carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) isotope values from multiple locations within a GLG (intratooth variation), from corresponding GLGs among teeth of an individual (intertooth variation), and from treated and untreated teeth.

**Results:** We observed no significant differences in the $\delta^{13}C$ or $\delta^{15}N$ values when sampling a single GLG from multiple locations (intratooth variation) or when comparing the same GLG across duplicate teeth of individuals (intertooth variation). One tooth in a triplicate set showed a significantly different but likely biologically inconsequential $\delta^{13}C$ value. Lastly, formic acid and graphite highlighting to accentuate GLGs did not significantly influence measured stable isotope values.

**Conclusions:** We validate several previous assumptions in this field of study. First, dentin samples for stable isotope analysis can be sampled from different locations across a GLG. Second, researchers can compare stable isotope values from the same GLGs of different teeth collected from the same individual in most cases, as the $\delta^{13}C$ and $\delta^{15}N$ values did not vary with the sampled tooth. Third, a common protocol of formic acid and graphite treatment to enhance GLG visibility does not bias the $\delta^{13}C$ and $\delta^{15}N$ values from dentin. We also describe factors to consider and cautions associated with these conclusions.

## 1 | Introduction

Teeth have been a useful tissue from which to measure stable isotope values of marine mammals for decades [1–4], as stable nitrogen ($\delta^{15}N$) and carbon ($\delta^{13}C$) isotopes are assimilated into tissues and can provide insights into feeding ecology and habitat use [5, 6]. Teeth are particularly useful for this purpose because they are metabolically inert and resistant to decomposition, which makes them ideal for long-term storage in museums. Additionally, teeth are often collected as part of a set, allowing for destructive sampling of one tooth without compromising the others, enabling detailed ecological analyses across an individual's life history. Furthermore, odontocetes (toothed whales such as belugas, porpoises, and dolphins) are monophyodonts, meaning they develop only one set of permanent teeth for their entire life [7]. These teeth are

characterized by annual, incremental dentin deposits, called growth layer groups (GLGs) [8–10], that gradually fill in the open pulp cavity for most of the animal's life. As a result, stable isotope analysis of metabolically inert dentin from GLGs allows for reconstruction of ecological chronologies over the life of the individual. Researchers can glean an overall age for the animal by sectioning the tooth in half longitudinally and counting GLGs [11–13]. With this method, specific years of interest can also be targeted for sampling [14].

The exact environmental drivers and physiological mechanisms by which GLGs are distinctly formed are as yet unclear (see discussions in [11, 15, 16]); however, it is known that GLGs in marine mammals grow horizontally in width as a whole layer during synthesis, as opposed to building vertically from pulp to crown or vice versa [17]. Although there is evidence by Walker and Macko [2] for isotopic homogeneity of dentin in a pinniped and a cetacean (see below) through subsampling a mixture from a whole single tooth, it has not, to our knowledge, been empirically demonstrated that stable isotope values are consistent along a given GLG. This is especially relevant for very large odontocete teeth such as those from sperm whales (*Physeter macrocephalus*) or killer whales (*Orcinus orca*) where an isotopic sample represents a very small fraction of an entire GLG. Additionally, pulp stones, cracks in the tooth, occlusion wear, or other factors may dictate where sampling can occur along a GLG and prevent using the same location across GLGs. One of our research goals was to examine stable isotope values taken from two different locations along a single GLG from a given killer whale tooth (i.e., closer to the root and closer to the crown), with the hypothesis that isotope values from both locations should be equivalent because GLGs form as a whole layer along the length of the tooth over the course of a year [14].

Walker and Macko [2] also present one of few studies that examined isotopic variation among teeth of an individual. They found very little isotopic variation in eight teeth from a walrus (*Odobenus rosmarus*; but see Stewart and Stewart [18], which did find age estimation differences among walrus teeth) and 12 teeth from a bottlenose dolphin (*Tursiops truncatus*). However, the sample size in Walker and Macko [2] was limited to one animal of each species, and they compared homogenized whole dentin among different teeth rather than among individual GLGs from the same tooth. Toothed whales have homodont dentition [19], meaning all teeth are structurally similar, but there can be some variation in that teeth located at the very anterior or posterior end of the jaw can be substantially smaller than those in the middle. In addition, dentin deposition in those smaller anterior or posterior teeth ceases at an earlier age for some cetacean species, such as bottlenose dolphins [20] and short-finned pilot whales [21] (*Globicephala macrorhynchus*), resulting in fewer GLGs in those teeth compared with teeth located in the center of the jaw. As a result, the straightest, largest teeth from the center of the jaw are likely most ideal for age estimation [22], but in some cases, only peripheral smaller or curved teeth are available for the destructive sampling necessary to estimate age and obtain dentin. A second goal of our research using killer whale teeth was to examine the potential for isotopic homogeneity of GLGs among teeth of an individual, again with the hypothesis that isotope values should be equivalent in corresponding GLGs among teeth, provided the GLGs are accurately matched across teeth.

Although GLGs of teeth are a powerful tool for biological and ecological insight, the GLGs themselves can be challenging to delineate. The presence of supernumerary or accessory lines can also complicate the task [9, 10, 23]. In order to visualize GLGs on the internal face of a half-tooth more clearly, the tooth halves are often "etched" in formic acid to produce raised ridges and grooves that define GLGs (reviewed in Read et al. [14], although accessory lines can be accentuated in this way as well). Then, a soft graphite pencil is lightly rubbed over the etched surface to highlight the ridges and further aid in GLG identification.

Etching and penciling is a common technique, but the potential effects of these treatments on stable isotope analysis of dentin are unclear. Numerous studies assumed that formic acid would not significantly influence stable isotope values, despite containing carbon ($HCO_2H$), because only the surface of the tooth is exposed, and the portion of dentin exposed to the acid is relatively small compared with the entirety of collected dentin for analysis [1, 24, 25]. Others have attempted to discard the "outer surface" (undefined in Hanson et al. [26], depth of 100 μm in Knox et al. [27]), although the depth to which formic acid might penetrate is unknown, and it is advantageous to sample as shallowly as possible to avoid layers underlying the target. Overall, either graphite has been removed before sampling, although it is uncertain if all the graphite was effectively removed, or the untreated tooth half was cross-referenced and sampled instead [28, 29]. This can still lead to concerns, as GLGs are less visible on untreated sections, and another source of error is introduced by aging one half of a tooth but sampling from the other.

To the best of our understanding at this time, Stukonytė et al. [30] present the only study that has investigated the effects of formic acid and graphite penciling on the stable isotope values of dentin. They found a significant effect of formic acid etching on sperm whale dentin from 30 teeth, leading to an increase of 0.2‰ for both $\delta^{13}C$ and $\delta^{15}N$ values. By cleaning graphite off of an etched tooth, they found no effect from the presence or absence of graphite. However, the authors caution against the universal application of their findings, given that treatment protocols (e.g., formic acid concentration and exposure time) can differ. In their case, 10% formic acid was used for a duration of 30 h. A third goal of our study also aimed to examine the effects of 10% formic acid and graphite on stable isotope analysis of dentin; however, we used killer whale teeth exposed for 12 h [25], less than half the time of Stukonytė et al. [30]. Additionally, our comparison framework differed (see Section 2), as we made comparisons of treated dentin (e.g., etched and penciled) to both untreated dentin and dentin with the 'contaminated' surface layer removed/cleaned.

Overall, our study aims to address three critical questions regarding stable isotope analysis of dentin from marine mammal teeth. First, we assess whether stable isotope values ($\delta^{13}C$ and $\delta^{15}N$) remain consistent across different locations within a single GLG. We hypothesize that isotope values will be uniform regardless of where within the GLG the sample is taken. Second, we investigate

whether isotope values from corresponding GLGs in different teeth of the same individual remain consistent, with the hypothesis that values will not vary significantly between teeth. Finally, we examine the influence of common pretreatment methods, acid etching and pencil highlighting, on stable isotope values. Specifically, we aim to determine whether these treatments affect the $\delta^{13}C$ and $\delta^{15}N$ values in comparison to untreated dentin samples. By addressing these questions, our study aims to refine methodological approaches in stable isotope analysis and enhance reproducibility in research using marine mammal dentin.

## 2 | Methods

In total, we used 37 killer whale teeth representing 24 unique individuals to examine our three objectives (Table S1). See Bowen and Kurle [31] for more detailed methods on tooth processing and age estimation, as we used these same killer whale specimens, protocols, and stable isotope data in separate studies with the foundations described there (see also Read et al. [14] for a more general review).

Briefly, whole teeth were longitudinally bisected on a modified Bosch® TC10 wet tile saw (Farmington Hills, MI, USA). The cut face of the teeth was polished by hand with 400-grit, then 600-grit, wet sandpaper disks to remove saw marks. We then placed the tooth cut face down into 10% formic acid [25] to a depth of 0.5–1 cm [32] for 12 h [25] to decalcify the tooth surface and allow the GLGs to become more visually apparent. After rinsing, soaking, and drying, we used a soft graphite pencil (6B) on the surface of the tooth face to highlight GLG ridges and grooves. We used an Epson WorkForce flatbed scanner to obtain high-resolution (800–1200 dpi) images (.tiff files) of all cut tooth faces, and we analyzed the images using GIMP (GNU Image Manipulation Program Version 2.10.6) to mark GLG boundaries for every tooth, although the physical tooth was consulted under a microscope in some cases for clarification if needed. All teeth were aged blindly (i.e., no reference to catalog number or other metadata, with the exception of BBM-J18 that was used as a known-age reference tooth) by K.B. across three independent sessions that were evaluated together to form a consensus. We were limited to one reader due to logistical constraints, and previous studies on aging teeth have also utilized one reader [28, 33] (see also Read et al. [14] for discussions on interreader and intrareader variation).

Finally, we collected dentin from targeted GLGs using a computer-controlled micromill (MicroMill2, Elemental Scientific Lasers, Omaha, NE, USA) at the NOAA Southwest Fisheries Science Center (La Jolla, CA). We used drill bits (Brasseler USA Dental Instruments, Savannah, GA, USA) that ranged from 300 to 1000 μm depending on the width of the targeted GLG. For a yield of approximately 2 mg of dentin for stable isotope analysis, our drill path was 8–10 mm in length and 200–350 μm in depth. If the GLG was relatively wide and warranted an 800- or 1000-μm bit, then we could drill a shorter and shallower path to obtain ~2 mg of powder. If the GLG was relatively narrow and required a 300- or 500-μm bit, we needed to drill a longer or deeper line to obtain the minimum amount of dentin. In either case, we subsampled approximately 1.2 mg of dentin from the total yield to package into 5 × 9 mm tin capsules for stable isotope analysis, reported in delta notation:

$$\delta^{13}C \text{ or } \delta^{15}N = \left[(R\text{sample} \div R\text{standard}) - 1\right] \times 1000$$

where $R$ represents the heavy:light ratio for $\delta^{13}C$ ($^{13}C/^{12}C$) or $\delta^{15}N$ ($^{15}N/^{14}N$) for the sample or an international standard as indicated. Standards were Vienna Peedee Belemnite (VPDB) and atmospheric nitrogen for measures of the $\delta^{13}C$ and $\delta^{15}N$ values, respectively.

Our samples were analyzed primarily in three laboratories: the University of California, Santa Cruz Stable Isotope Laboratory using a CE Instruments NC2500 elemental analyzer coupled to a Thermo Scientific DELTAplus XP isotope ratio mass spectrometer via a Thermo Scientific ConFlo III; the University of California, Davis, Stable Isotope Facility using a PDZ Europa ANCA-GSL elemental analyzer coupled to a PDZ Europa 20–20 isotope ratio mass spectrometer; and the University of New Mexico Center for Stable Isotopes using a Costech 4010 elemental analyzer coupled to a Thermo Scientific Delta V mass spectrometer via a Thermo Scientific ConFlo IV. Four of the 161 total samples were analyzed at the University of Florida Light Stable Isotope Mass Spec Lab using a Carlo Erba elemental analyzer coupled to a Thermo Delta V Advantage isotope ratio mass spectrometer via a Thermo Scientific ConFlo II. The laboratories used a variety of reference standards intermittently for quality control, but in all runs, the standard deviations (SDs) were ≤ 0.3‰ for $\delta^{13}C$ values and ≤ 0.4‰ for $\delta^{15}N$ values. In most cases, the same laboratory analyzed paired samples, which were compared for each of our objectives.

Protocols and statistical analyses, all conducted in R [34] interfaced with R Studio [35], for our three objectives are described below.

### 2.1 | Do the $\delta^{13}C$ and $\delta^{15}N$ Values Measured From a GLG Remain Consistent Regardless of the Area Within the GLG From Which Dentin Is Sampled?

To test this question, we took two samples (an "original" sample and a "replicate" sample) from different points along a single GLG (Figure 1A). Due to GLG compaction at the root and crown of the tooth and an increased risk of drilling into an underlying layer, we took the two samples from the middle of the section of the tooth when possible, so one sample would not be more likely to contain nontarget GLGs than the other. We did this for eight different GLGs, for a total of 16 samples, using seven unique teeth. We sampled a range of GLGs representing age 4 to age 19 of the animal's life.

For the collection of this dataset, to reduce formic acid and graphite presence, we discarded the surface dentin to a depth of ~25 μm using compressed air, with all samples treated equally for comparison of the stable isotope values from different locations of a single GLG (see Objective III for testing of formic acid and graphite effects). The $\delta^{13}C$ and $\delta^{15}N$ values for both the original and the replicate groups appeared consistent with normal distributions via the Shapiro–Wilk test, $W(8) = 0.9$ ($p > 0.2$ for

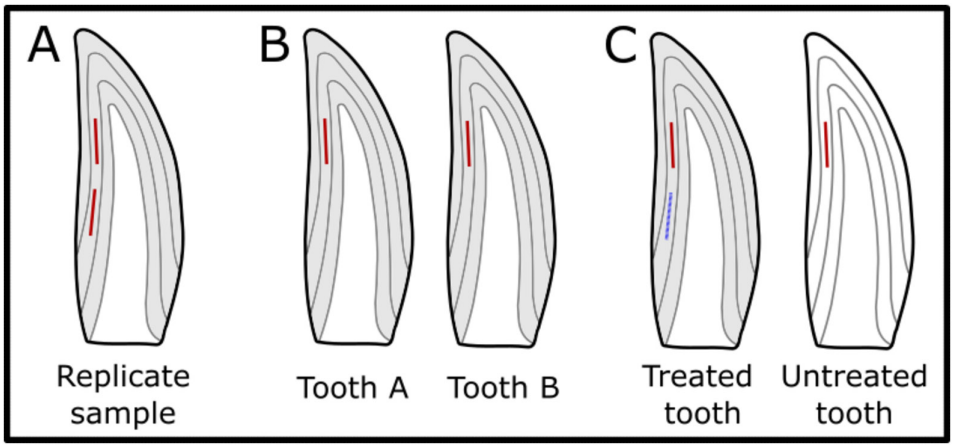


**FIGURE 1** | Sampling schemes for the three objectives of our study illustrated with the cut surface of tooth halves and visible growth layer groups (GLGs). A gray backdrop indicates the tooth half has been etched and penciled, with a white center indicating an open pulp cavity. Our first objective (A) was to sample two locations within the same GLG of an etched and penciled tooth half, where the solid red lines indicate a representative drill sampling path. Our second objective (B) was to sample the same GLG in two different teeth (Tooth A and Tooth B) from the same individual. Our third objective (C) was twofold: (i) to compare a sample from a GLG with formic acid and graphite intact (red solid line on gray backdrop) to a sample from the same GLG with the formic acid and graphite cleaned away (blue dashed line) and (ii) to compare a sample from a treated GLG with formic acid and graphite intact (red solid line on gray backdrop) to a sample from the same GLG on the other untreated tooth half (red solid line on a white backdrop).

all), so we proceeded with parametric paired *t*-tests to assess statistical significance of the mean difference between the original and replicate samples.

### 2.2 | Do the $\delta^{13}C$ and $\delta^{15}N$ Values Measured in Dentin From a Specific GLG (e.g., the GLG That Represents a Specific Year of the Animal's Life) Remain Consistent Regardless of the Tooth From Which the GLG Is Sampled?

We sampled 106 GLGs total across 12 pairs of teeth to investigate isotopic homogeneity of GLGs among teeth within an individual (Figure 1B and Table S2). The number of GLGs that we sampled per tooth ranged from one to eight, with the youngest age being 4 and the oldest age being 25. Note that we sampled four teeth from specimen USNM-594671 but treated them as two independent pairs (i.e., Pair 1 and Pair 2 in Table S2) rather than one quadruplicate set. Each pair had a different original catalog number (i.e., NMML-82 and MML-1966-145) as they were thought to be different specimens, though were later discovered after sampling to be teeth from the same single individual. As a consequence, we did not sample the same corresponding GLGs across all four teeth for fair statistical comparison, only the same corresponding GLGs for each pair. Nonetheless, we retained both pairs in our dataset because they represented a notable comparison between a pair of teeth directly adjacent (Pair 1) and a pair of teeth relatively far apart (Pair 2).

The outermost dentin was again discarded with compressed air to a depth of ~25 μm for this protocol. We separated the stable isotope measurements of GLGs for "Tooth A" and those for "Tooth B" for each pair, representing teeth from two different locations in the jaw of a single animal. We were unable to make systematic comparisons of tooth location across all individuals (e.g., collect one middle tooth and one peripheral tooth for every individual, or one left tooth and one right tooth for every individual), but we report tooth positions (and the approximate length of the tooth for a size comparison reference) when known in Table S2. We again examined the $\delta^{13}C$ and $\delta^{15}N$ values of the paired samples from corresponding GLGs for normality using the Shapiro–Wilk test. The test showed evidence of non-normality for the $\delta^{13}C$ values of the Tooth A group, $W(53) = 0.95$, ($p < 0.01$) and for the $\delta^{15}N$ values of the Tooth B group, $W(53) = 0.92$ ($p < 0.01$), so we used a conservative approach with the nonparametric Wilcoxon signed-rank test to compare the difference between group means.

We also had access to a single triplicate set of teeth from one individual killer whale and examined this specimen separately. We measured the same five GLGs in all three teeth. The $\delta^{13}C$ values reflected a normal distribution, $W(5) = 0.9$ ($p > 0.3$ for all), and the $\delta^{15}N$ values reflected a normal distribution for two of the three teeth, $W(5) = 0.8$ ($p > 0.05$ for both), with one sample showing mild nonnormality, $W(5) = 0.7$ ($p = 0.04$). Given that ANOVAs are relatively robust to nonnormality [36], we used a repeated-measures ANOVA to compare the stable isotope values of the five GLGs measured in all three teeth.

### 2.3 | What Are the Effects of Acid Etching and Pencil Highlighting on Stable Isotope Values of Dentin in Comparison to (a) Dentin With Removal of the Contaminants and (b) Dentin Never Exposed to the Contaminants?

For Scenario (a), the effect of removing the contaminants from the tooth dentin compared with a treated tooth with potential contaminants intact, we utilized four teeth (with two teeth from individual USNM-594671; Table S1), where a total of 10 GLGs were sampled twice: a "cleaned" sample in which the outer

surface dentin to a depth of ~25 μm was discarded to reduce the presence of formic acid and graphite and an "uncleaned" (or potentially contaminated) sample in which no dentin was discarded and any surface formic acid and graphite were retained (Figure 1C). Thus, we had 20 samples total. Shapiro–Wilk tests showed a significant departure from normality for the $\delta^{15}$N values of the cleaned and uncleaned groups, $W(10) = 0.8$ ($p < 0.05$ for both), but not for the $\delta^{13}$C values, $W(10) = 0.9$ ($p > 0.07$). Accordingly, we used the nonparametric Wilcoxon signed-rank test to compare the $\delta^{15}$N values and the parametric paired *t*-test for the $\delta^{13}$C values.

For Scenario (b), the effect of contaminant removal compared with an untreated tooth, we examined two GLGs within each of five teeth. Each GLG was sampled from paired tooth halves, one sample treated with formic acid and graphite under the "clean" condition (i.e., with formic acid and graphite from the first ~25 μm discarded) and the other collected from the corresponding tooth half never exposed to the potential contaminants, for a total of 20 samples (Figure 1C). We still discarded the outermost ~25 μm of dentin from the untreated tooth half before collecting the sample to reduce influence from other external contamination such as dust and human oils. Although it is more difficult to read GLGs from untreated tooth halves and ensure the same GLG is being sampled when compared with the treated half, we did intentionally select teeth and specific GLGs with identifying features such that we could be confident about correctly cross-referencing tooth halves. For example, we could use the presence of a pulp stone (a distinct calcified mass embedded in the dentin) within the tooth as a feature to help guide GLG identification. Shapiro–Wilk tests showed evidence of normal distributions for both $\delta^{13}$C and $\delta^{15}$N values of treated and untreated groups, $W(10) = 0.9$ ($p > 0.05$ for all), so we used paired *t*-tests to determine if there were differences in isotopic values from dentin apparently cleaned of contaminants and dentin never exposed to the contaminants.

## 3 | Results

### 3.1 | Do the $\delta^{13}$C and $\delta^{15}$N Values Measured From a GLG Remain Consistent Regardless of the Area Within the GLG From Which Dentin Is Sampled?

The paired *t*-tests did not detect a significant difference between original and replicate stable isotope measurements within a single GLG (Figure 1A) for either the $\delta^{13}$C values, $t(7) = -0.96$ ($p = 0.37$), or the $\delta^{15}$N values, $t(7) = -0.035$, ($p = 0.97$). The mean (±SD, hereafter) $\delta^{13}$C values for the original and replicate samples were $-13.8 \pm 2.0$‰ and $-13.8 \pm 2.1$‰, respectively. The mean $\delta^{15}$N values for the original and replicate samples were $16.5 \pm 2.3$‰ and $16.5 \pm 1.3$‰, respectively. We also calculated the difference between the original and replicate stable isotope values for each pair and then calculated the mean, SD, and range of the differences. The mean differences were more constrained for the $\delta^{13}$C values than for the $\delta^{15}$N values ($\delta^{13}$C: $0.1 \pm 0.1$‰; $\delta^{15}$N: $1.1 \pm 1.4$‰), although there were notable differences in the $\delta^{15}$N values between paired samples for two of eight comparisons (2.9‰, 3.7‰). Summary statistics for all objectives are provided in Table 1.

### 3.2 | Do the $\delta^{13}$C and $\delta^{15}$N Values Measured in Dentin From a Specific GLG (e.g., the GLG That Represents a Specific Year of the Animal's Life) Remain Consistent Regardless of the Tooth From Which the GLG Is Sampled?

The Wilcoxon signed-rank tests indicated that there were no statistically significant differences in the $\delta^{13}$C ($Z = -1.14$, $p = 0.25$) or $\delta^{15}$N values ($Z = -0.65$, $p = 0.52$) measured between Tooth A and Tooth B from the same individual for 12 pairs (Figure 1B and Table 1). The mean $\delta^{13}$C value (±SD) of all GLGs was the same for both the Tooth A and Tooth B datasets ($-13.2 \pm 1.0$‰), and the mean $\delta^{15}$N values for Tooth A and B groups were $17.5 \pm 0.9$‰ and $17.5 \pm 1.3$‰, respectively. Mean differences in the stable isotope values between Tooth A and Tooth B were less than 0.5‰ for both the $\delta^{13}$C ($0.2 \pm 0.1$‰) and the $\delta^{15}$N values ($0.4 \pm 0.5$‰). Again, there was a greater range in the $\delta^{15}$N values (3.4‰) than in the $\delta^{13}$C values (0.5‰), although 50/53 measurements showed a difference of less than 1.0‰.

We noted our use of four teeth from specimen USNM-594671, although we could only compare two pairs separately because we were unable to sample the same GLGs across all four teeth. The only exception was GLG 19, which we did measure from all four teeth, although the sample size is too small for statistical comparison. However, we did observe that the $\delta^{13}$C values for GLG 19 among all four teeth were between −13.3 and −13.6‰ (SD = 0.2‰), and the $\delta^{15}$N values were between 17.8 and 18.9‰ (SD = 0.5‰).

The repeated-measures ANOVA for the triplicate set of teeth also did not detect a significant difference among the $\delta^{15}$N values of the teeth, $F_{(2,12)} = 3.43$ ($p = 0.08$; mean values from $18.7 \pm 0.3$‰ to $19.0 \pm 0.4$‰). When comparing the same GLG across the three teeth, differences in the $\delta^{15}$N values ranged between 0.1‰ and 0.5‰, with an average of 0.3‰. Unlike the $\delta^{15}$N values, there was a significant difference among the $\delta^{13}$C values, $F_{(2,12)} = 8.26$ ($p = 0.01$). The mean $\delta^{13}$C values for each of the three teeth were (1) $-13.7 \pm 0.4$‰, (2) $-13.6 \pm 0.2$‰, and (3) $-13.4 \pm 0.2$‰. The largest range among triplicate comparisons of each GLG was 0.7‰ (0.2‰ minimum, 0.7‰ maximum), with an average of 0.4‰. Post hoc paired *t*-tests indicated that the third tooth was driving the global significance, Tooth 1 to Tooth 3: $t(4) = 3.3$ ($p = 0.03$); Tooth 2 to Tooth 3: $t(4) = 3.7$ ($p = 0.02$).

### 3.3 | What Are the Effects of Acid Etching and Pencil Highlighting on the Stable Isotope Values of Dentin From Killer Whale Teeth in Comparison to (a) Dentin With Removal of These Potential Contaminants and (b) Dentin Never Exposed to the Contaminants?

For Scenario (a), there was no significant difference in the five $\delta^{15}$N values of cleaned (i.e., contaminants removed; mean of $18.3 \pm 1.3$‰) and five uncleaned (i.e., contaminants present; mean of $18.2 \pm 1.2$‰) dentin samples (Figure 1C and Table 1) via the Wilcoxon signed-rank test ($Z = -1.27$, $p = 0.20$). Likewise, there was no significant difference between the mean $\delta^{13}$C

**TABLE 1** | Summary statistics (mean, standard deviation [SD], and range [max, min]) for the three objectives of this study (Figure 1). Object I was to compare stable isotope values from different locations within a single growth layer group (GLG). Object II was to compare stable isotope values from two different teeth within the jaw of a single individual (note that the individual we measured in triplicate is excluded here, see main text). Objective IIIa was to compare stable isotope values from "clean" and "unclean" (i.e., formic acid residue and graphite present) tooth halves, and Objective IIIb was to compare "clean" and "untreated" (i.e., never exposed to formic acid or graphite) tooth halves. Sample sizes ($n$) represent the number of GLGs from which dentin was extracted for stable isotope analysis. For each category, we also report the summary statistics for the difference in stable isotope values between each paired sample.

| | $\delta^{13}$C (‰) | | | $\delta^{15}$N (‰) | | |
|---|---|---|---|---|---|---|
| | **Mean** | **SD** | **Range (min, max)** | **Mean** | **SD** | **Range (min, max)** |
| Objective I ($n=16$) | | | | | | |
| Original ($n=8$) | −13.8 | 2.0 | 5.5 (−17.2, −11.7) | 16.5 | 2.3 | 7.4 (11.8, 19.2) |
| Replicate ($n=8$) | −13.8 | 2.1 | 5.9 (−17.5, −11.6) | 16.5 | 1.3 | 3.6 (15.0, 18.6) |
| Difference | 0.1 | 0.1 | 0.3 (0.0, 0.3) | 1.1 | 1.4 | 3.5 (0.2, 3.7) |
| Objective II ($n=106$) | | | | | | |
| Tooth A ($n=53$) | −13.2 | 1.0 | 3.6 (−15.3, −11.6) | 17.5 | 0.9 | 4.3 (15.3, 19.6) |
| Tooth B ($n=53$) | −13.2 | 1.0 | 4 (−15.4, −11.4) | 17.5 | 1.3 | 7.0 (13.0, 20.0) |
| Difference | 0.2 | 0.1 | 0.5 (0.0, 0.6) | 0.4 | 0.5 | 3.4 (0.0, 3.4) |
| Objective IIIa ($n=10$) | | | | | | |
| Clean ($n=5$) | −13.1 | 0.5 | 1.6 (−13.6, −12.0) | 18.3 | 1.3 | 4.7 (16.7, 21.5) |
| Unclean ($n=5$) | −13.1 | 0.4 | 1.5 (−13.6, −12.1) | 18.2 | 1.2 | 4.5 (16.7, 21.2) |
| Difference | 0.1 | 0.1 | 0.3 (0.0, 0.3) | 0.2 | 0.1 | 0.4 (0.0, 0.4) |
| Objective IIIb ($n=20$) | | | | | | |
| Clean ($n=10$) | −12.3 | 1.3 | 3.2 (−14.5, 11.3) | 18.6 | 1.3 | 3.9 (16.9, 20.8) |
| Untreated ($n=10$) | −12.8 | 1.1 | 2.8 (−14.0, −11.1) | 18.4 | 1.1 | 2.9 (17.0, 19.9) |
| Difference | 0.4 | 0.3 | 0.9 (0.0, 0.9) | 0.4 | 0.3 | 0.8 (0.1, 0.9) |

values via the paired $t$-test, $t(9)=0.44$, $p=0.67$. The mean $\delta^{13}$C value of the cleaned ($n=5$ GLGs) and uncleaned ($n=5$ GLGs) samples was $-13.1\pm0.5$‰ and $-13.1\pm0.4$‰, respectively. The mean and range of differences in stable isotope values between pairs were small (mean $\delta^{13}$C value $=0.1\pm0.1$‰, range $=0.3$‰; mean $\delta^{15}$N value $=0.2\pm0.1$‰, range $=0.8$‰).

For Scenario (b), paired $t$-tests also showed no evidence of significant differences between dentin that had been cleaned of contaminants ($n=10$ GLGs) and dentin that had never been exposed ($n=10$ GLGs) for either the $\delta^{13}$C values, $t(9)=0.87$ ($p=0.41$), or $\delta^{15}$N values, $t(9)=-0.90$ ($p=0.40$). Mean $\delta^{13}$C and $\delta^{15}$N values for the teeth cleaned of potential contaminants were $-12.8\pm1.1$‰ and $18.4\pm1.1$‰, compared with $-12.9\pm1.3$‰ and $18.6\pm1.3$‰ for the untreated teeth, respectively. Finally, the mean ($\delta^{13}$C value and $\delta^{15}$N value $=0.4\pm0.3$‰) and range ($\delta^{13}$C value $=0.9$; $\delta^{15}$N value $=0.8$‰) of differences between paired samples were slightly greater than comparisons between cleaned and uncleaned samples (Scenario a).

## 4 | Discussion

In this study, we empirically tested three common assumptions underlying stable isotope analysis of marine mammal dentin. We found, generally, that neither the $\delta^{13}$C nor the $\delta^{15}$N values of dentin are dependent upon which region of a given GLG or which specific tooth is sampled. Likewise, pretreating a tooth with 10% formic acid and graphite highlighting for GLG enhancement does not bias the $\delta^{13}$C or $\delta^{15}$N values. However, our results may be specific to the conditions we tested here. We discuss our findings and potential limitations in more detail below.

### 4.1 | Do the $\delta^{13}$C and $\delta^{15}$N Values Measured From a GLG Remain Consistent Regardless of the Area Within the GLG From Which Dentin Is Sampled?

Our results support the hypothesis that a given GLG is isotopically uniform throughout a tooth. This might be expected because dentin (representing stable isotopes derived from the diet) is uniformly synthesized as a layer spanning the length of the tooth during GLG formation [11], rather than a progression from the root to the crown that would lead to differing stable isotope values depending on the animal's diet at the time. In the latter case, stable isotope measurements could be dependent on where, within the GLG, a sample was taken. However, even with the understanding that GLGs form as a whole layer over the span of a year, dentin is a complex structure

composed of both mineralized inorganic material and organic collagen. The mineral concentrations in dentin may not be equal throughout a GLG, in part due to the presence and variability of calcospherites (hydroxyapatite or calcium apatite crystals) as well as secondary mineralization by calcium salts [11]. In bone, organic collagen is typically isolated from the inorganic matrix via decalcification [37, 38] for stable isotope measurements, but studies have shown that this process is not necessary for modern marine mammal teeth and does not affect isotope values [39, 40]. Treatment is likely not necessary because teeth contain very little lipid compared with bone, and inorganic carbonate in dentin may have a similar stable isotope range as collagen [39, 40]. Researchers should still consider the atomic C:N ratio of pure collagen, which is between 2.9 and 3.6 [37, 41], as values outside this range may indicate contaminated collagen (see Bowen and Kurle [42] for treatment recommendations of killer whale bone). In any case, we found that the potential sources of heterogeneity in dentin were not sufficient to affect the stable isotope values measured in different sections of a GLG.

Another potential factor to consider when sampling a GLG is the concentric nature of the layers and the likelihood of drilling into layers below the target. Because layers are compressed and thinner at the root and the crown of the tooth, samples taken from those regions may need to be shallower than samples taken at the middle of the tooth to avoid underlying layers. In our experimental design, we attempted to control for this issue by drilling two locations in a GLG (to the same depth) that could still be described as the middle region of the tooth. We have found evidence of isotopic ontogenetic changes in adult killer whales [31], meaning that accidentally sampling a deeper (i.e., older) GLG could significantly bias the data and interpretations. So while our data suggest there is leniency in the exact sampling point of a GLG, it is advisable to sample from the middle of the tooth when possible or adapt the drill path to be longer but shallower if the sample needs to be taken from the peripheral root or crown regions.

### 4.2 | Do the $\delta^{13}C$ and $\delta^{15}N$ Values Measured in Dentin From a Specific GLG (e.g., the GLG That Represents a Specific Year of the Animal's Life) Remain Consistent Regardless of the Tooth From Which the GLG Is Sampled?

Overall, our results support that stable isotope values measured from dentin taken from GLGs that represent the same year are not dependent on the exact tooth from which each GLG is sampled. We had 12 pairs of teeth in our dataset, which represented a diversity of position comparisons because we were unable to consistently sample the same two teeth from every individual (Table S2). However, where tooth position was known, most pairs of teeth originated from near the middle of the jaw and were one to three positions apart. We did include one pair of teeth (the aforementioned specimen ID USNM-594671) that were nearly as far apart as possible, yet the $\delta^{13}C$ and $\delta^{15}N$ values remained similar (Table S2).

There was one tooth in a triplicate set that was the only exception to our finding of consistent stable isotope measurements in the same GLGs between teeth, although the differences in the average $\delta^{13}C$ values were only 0.2‰ or 0.3‰ below those of the other two teeth. The largest difference in the $\delta^{13}C$ value of a given GLG among the three teeth was only slightly greater than the average at 0.7‰. Given that biologically meaningful increments of $\delta^{13}C$ values to infer animal habitat use and feeding patterns are ~1.0‰–2.0‰ [6, 43–45], the differences of 0.2‰–0.3‰ we observed are likely not biologically significant. The locations of these three teeth (e.g., maxilla or mandible, left or right, and posterior or anterior; see Table S2) were not noted, so we were unable to draw inferences about whether or not position might have influenced the outlying values.

An important potential source of error in testing the isotopic consistency of GLGs between teeth of an individual is correctly matching GLGs. Although stable isotope values were not examined, several studies have given attention to whether or not age estimations vary with different teeth from one individual [20, 21, 23, 46, 47]. If age estimations differ by tooth, the factors that contribute to these differences need to be considered when matching GLGs. For example, Hui [20] found different GLG counts within teeth from bottlenose dolphins because the anterior teeth cease dentin deposition at 10–12 years old, but other teeth showed more than double that count. Kasuya and Matsui [21] similarly found that the anterior and posterior teeth of short-finned pilot whales showed significantly fewer GLGs than teeth from the middle of the jaw, even if the pulp cavity was still open. In contrast, other studies have described similar GLG counts among different teeth from an individual for sperm whales, spinner dolphins (*Stenella longirostris*), pantropical spotted dolphins (*Stenella attenuate*), common dolphins (*Delphinus delphis*), bottlenose dolphins, and long-finned pilot whales (*Globicephala melas*) [23, 46, 47].

We found that the killer whale teeth within individuals in our study did generally reflect consistent GLG counts. However, we also found that teeth located in the anterior and posterior sections of the mouth appeared to cease dentin deposition earlier than those positioned in the middle of the mouth due to the closure of the pulp cavity. For example, we counted the same number of GLGs (~25) in a posterior tooth with a nearly closed cavity as we did for a middle tooth with an open pulp cavity still comprising approximately 20% of the cut tooth surface area (the aforementioned specimen ID USNM-594671; Figure 2). The matching GLGs between these two teeth could be compared, but we would not have been able to sample older GLGs from the posterior tooth that were only present in the middle tooth. Indeed, we suspect age ~25 may be the point at which the most posterior teeth fill in, as we found much older animals (~45 GLGs in the middle teeth) with about half as many GLGs in the most posterior tooth (Bowen, pers. obs.). For that reason, we caution against using very anterior or posterior teeth for aging or stable isotope analysis. Unfortunately, the positions of the teeth in our triplicate set were unidentified, but all three were approximately the same size with a similar degree of exposed pulp cavity, meaning an anterior or posterior position in the mouth was unlikely to be a contributing factor to the small but measurable difference in $\delta^{13}C$ values.

To summarize, we found that GLG counts were consistent across teeth within an individual and believe GLG matching


**FIGURE 2** | A high-resolution, scanned image of bisected teeth from killer whale specimen ID USNM-594671. The teeth have been etched in 10% formic acid and lightly penciled with graphite to highlight the growth layer groups (GLGs). Tooth A (top) was labeled as "Upper Left—12," meaning it was the 12th tooth in the jaw (i.e., generally the most posterior position). Tooth B (bottom) from the same animal was labeled as "Lower Right—7," meaning it was the seventh tooth in the jaw (i.e., centralized position). Both teeth produced GLG estimates of ~25, despite showing notably different lengths and pulp cavity sizes. (Note that the residual, chalk-like red surface material on Tooth B from a previous study was removed when we discarded the top ~25 μm of dentin, and no outlier stable isotope values were observed from this tooth. No other teeth in this study were marked with the red surface material.)

between two teeth was not a significant source of error contributing to our study, although we acknowledge this should be a consideration when comparing multiple teeth. Our data suggest there is leniency when choosing a particular tooth from which to sample for stable isotope analyses, but researchers should still prioritize a tooth located in the middle of the mouth when possible or otherwise take into account species-specific variation in tooth morphology or GLG patterns. If a killer whale tooth with a closed cavity is collected from the anterior or posterior portion of the mouth for sampling, then the age estimate and isotopic data may not reflect the animal's entire lifetime or an accurate age estimation.

### 4.3 | What Are the Effects of Acid Etching and Pencil Highlighting on the Stable Isotope Values of Dentin From Killer Whale Teeth in Comparison to (a) Dentin With Removal of These Potential Contaminants and (b) Dentin Never Exposed to the Contaminants?

Our data support that acid etching and pencil highlighting did not significantly affect the $\delta^{13}C$ or $\delta^{15}N$ values of killer whale teeth. We showed this result through comparisons of the same GLGs under two conditions: (a) an "uncleaned" sample that was unaltered and a "cleaned" sample in which formic acid and graphite from the surface had been discarded and (b) an uncleaned sample from the tooth treated with formic acid and graphite and a sample from the other tooth half that had never been exposed to the chemicals. In both cases, stable isotope values did not differ between the compared samples.

Our results partly agreed with those from Stukonytė et al. [30], the only other published study on the effects of formic acid and graphite on teeth from a marine mammal, as they also found no significant effect of graphite on the $\delta^{13}C$ or $\delta^{15}N$ values of dentin. The authors hypothesize that the amount of graphite is negligible in comparison to the amount of dentin sampled, and the graphite carbon signature is not detectable. For sperm whale and killer whale teeth, removing the graphite layer before collecting dentin for stable isotope analysis does not appear to be necessary.

Although our conclusions on the effect of graphite were similar, our results for the effect of formic acid on stable isotope values differed from those of Stukonytė et al. [30]: They found an increase of 0.2‰ on average for both the $\delta^{13}C$ (difference in minimum = 0.2‰, difference in maximum = 0.5‰) and $\delta^{15}N$ (difference in minimum = 0.6‰, difference in maximum = 0.1‰) values in sperm whale teeth etched with formic acid compared with untreated teeth, whereas we found no difference in isotope values for killer whale teeth. A notable change in our methodology was the amount of time the teeth were exposed to formic acid. The sperm whale teeth were soaked for 30 h compared with the killer whale teeth in the current study that soaked for 12 h. Thus, it could be advantageous to limit the duration of the formic acid exposure when targeting GLGs for stable isotope analyses. Teeth are etched with formic acid to expose the GLGs more clearly, and etching time is a trade-off between soaking the tooth long enough to obtain clear GLGs and short enough to avoid overetching, in which the GLGs may be obscured from decalcification activity. Evans and Robertson [32] found that ideal etching time was significantly related to the size of the tooth for sperm whales. In our study, we etched all sizes of killer whale teeth from ~5 to ~15 cm in length for 12 h and qualitatively found equally readable GLGs. Although Stukonytė et al. [30] did not specify the size of the sperm whale teeth used, killer whale and sperm whale teeth can be comparable [48] (~10–15 cm in length; Figure 2; Bowen, pers. obs.). Researchers should consider optimizing the formic acid etching time of their species and tooth sizes to be as short as possible while still yielding clear GLGs. The concentration of the formic acid is important to consider as well [32], although both our study and that of Stukonytė et al. [30] used 10% formic acid.

Another variable that could influence the effect of formic acid on stable isotope values in dentin is the depth of formic acid penetration given the permeability of the tooth [11]. Klevezal [11] remarks that lower concentrations and smaller sizes of calcospherites can increase permeability. It could be possible that certain species are prone to higher or lower levels of dentin permeability. Relatedly, the depth to which a GLG is sampled or the overall amount of dentin collected could affect whether or not formic acid biases stable isotope measurements. Stukonytė et al. [30] suggest that 0.9 mg of dentin was extracted and packaged for stable isotope analysis. In contrast, we collected up to approximately 2 mg of dentin and subsampled 1.2 mg for isotope analysis. As in the case with graphite, formic acid may comprise a negligible proportion of the dentin sample if 2 mg is collected, but the effect could differ if only 1 mg is collected because the formic acid could be more prevalent in the sample.

Although Stukonytė et al. [30] report a significant increase of 0.2‰ for both $\delta^{13}C$ and $\delta^{15}N$ values in sperm whale teeth from

formic acid, it is also important to consider biological context for interpretation. As previously noted, meaningful $\delta^{13}C$ increments are typically on the order of ~1‰–2‰ [6, 43–45]. Additionally, 0.2‰ was within our range of stable isotope measurement precision. Stukonytė et al. [30] examined a slightly larger sample size ($n = 30$ independent samples) than presented here ($n = 20$ independent samples total for Scenarios a and b), so it is possible we could have detected an effect of formic acid with additional specimens. However, our data suggest that killer whale teeth of any size (excluding those from young calves) can be etched in 10% formic acid for 12 h, highlighted with graphite pencil, and directly sampled for stable isotope analysis. For good practice, we still recommend discarding the outermost layer of dentin if possible due to any number of surface contamination possibilities. However, this may not be feasible for very narrow GLGs representing the oldest years of a killer whale's life, because all drilled dentin may be needed to have sufficient material for isotope analysis and to maintain the shallowest path possible so as to avoid underlying nontarget GLGs. Researchers studying smaller cetacean or pinniped teeth could encounter this same dilemma. Additional studies of different formic acid conditions across species may help establish a framework that can be used for pretreatment, age estimation, and stable isotope sampling of mammal teeth.

## 5 | Conclusions

Our study demonstrates that researchers utilizing dentin from GLGs of teeth can exercise some flexibility in their sampling approach. GLGs can be sampled across different regions of the tooth if cracks or other imperfections impede consistency, provided that care is taken to avoid drilling into an underlying GLG. If a study requires sampling teeth from different individuals, the teeth may be taken from different positions in the jaw if necessary. Finally, we showed here that, when following the protocol we described, researchers can continue the long-standing practice of acid etching and penciling a tooth's cut surface to enhance GLG visibility, without compromising stable isotope data. Future work is needed to determine if different combinations of acid concentration and soak duration than those used here might influence stable isotope values. Furthermore, additional comprehensive studies on how GLG counts (i.e., age estimation) may vary with each tooth in a single jaw would benefit this field, and there could be species-specific differences to investigate as well. Understanding GLG formation and boundaries is crucial when attempting to sample the same GLG among different teeth for stable isotope values or other analyses.

### Author Contributions

**Kelly R. Bowen:** conceptualization, data curation, formal analysis, investigation, resources, software, writing – original draft, writing – review and editing. **Carolyn M. Kurle:** conceptualization, funding acquisition, project administration, resources, supervision, writing – review and editing.


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### Data Availability Statement

The data that support the findings of this study are partially available in the Supporting Information of this article. Other data are available on request from the authors.

### Peer Review

The peer review history for this article is available at https://www.webofscience.com/api/gateway/wos/peer-review/10.1002/rcm.9997.

### References


1. K. A. Hobson and J. L. Sease, "Stable Isotope Analyses of Tooth Annuli Reveal Temporal Dietary Records: An Example Using Steller Sea Lions," *Marine Mammal Science* 14, no. 1 (1998): 116–129, https://doi.org/10.1111/j.1748-7692.1998.tb00694.x.

2. J. L. Walker and S. A. Macko, "Dietary Studies of Marine Mammals Using Stable Carbon and Nitrogen Isotopic Ratios of Teeth," *Marine Mammal Science* 15, no. 2 (1999): 314–334, https://doi.org/10.1111/j.1748-7692.1999.tb00804.x.

3. S. D. Newsome, M. T. Clementz, and P. L. Koch, "Using Stable Isotope Biogeochemistry to Study Marine Mammal Ecology," *Marine Mammal Science* 26, no. 3 (2010): 509–572, https://doi.org/10.1111/j.1748-7692.2009.00354.x.

4. C. R. Teixeira, G. C. Troina, F. G. Daura-Jorge, P. C. Simões-Lopes, and S. Botta, "A Practical Guide on Stable Isotope Analysis for Cetacean Research," *Marine Mammal Science* 38, no. 3 (2022): 1200–1228, https://doi.org/10.1111/mms.12911.

5. M. J. DeNiro and S. Epstein, "Influence of Diet on the Distribution of Carbon Isotopes in Animals," *Geochimica et Cosmochimica Acta* 42, no. 5 (1978): 495–506, https://doi.org/10.1016/0016-7037(78)90199-0.

6. M. J. Deniro and S. Epstein, "Influence of Diet on the Distribution of Nitrogen Isotopes in Animals," *Geochimica et Cosmochimica Acta* 45, no. 3 (1981): 341–351, https://doi.org/10.1016/0016-7037(81)90244-1.

7. M. D. Uhen, "Evolution of Dental Morphology (Cetacean)," in *Encyclopedia of Marine Mammals* (Academic Press, 2009), 316–318.

8. M. Nishiwaki and T. Yagi, "On the Age and the Growth of Teeth in a Dolphin (*Prodelphinus caeruleo-albus*)," *Scientific Reports of the Whales Research Institute* 8 (1953): 133–146.

9. V. B. Scheffer and A. C. Myrick, "A Review of Studies to 1970 of Growth Layers in the Teeth of Marine Mammals," *Report of the International Whaling Commission* 3, no. special issue (1980): 51–63.

10. A. A. Hohn, M. D. Scott, R. S. Wells, J. C. Sweeney, and A. B. Irvine, "Growth Layers in Teeth From Known-Age, Free-Ranging Bottlenose Dolphins," *Marine Mammal Science* 5, no. 4 (1989): 315–342, https://doi.org/10.1111/j.1748-7692.1989.tb00346.x.

11. G. A. Klevezal, *Recording Structures of Mammals: Determination of Age and Reconstruction of Life History*, 1st ed. (Routledge, 1995), https://doi.org/10.1201/9780203741146.

12. W. F. Perrin and A. C. Myrick, "Age Determination of Toothed Whales and Sirenians," in *International Conference on Determining Age of Odontocete Cetaceans (and Sirenians)* (International Whaling Commission, 1980).

13. A. C. Myrick, P. Yochem, and L. Cornell, "Toward Calibrating Dentinal Layers in Captive Killer Whales by Use of Tetracycline Labels," *Rit Fiskideildar* 11 (1988): 285–296.

14. F. L. Read, A. A. Hohn, and C. H. Lockyer, "A Review of Age Estimation Methods in Marine Mammals With Special Reference to Monodontids," *NAMMCO Scientific Publications* 10 (2018): 1–67, https://doi.org/10.7557/3.4474.

15. I. L. Boyd and J. P. Roberts, "Tooth Growth in Male Antarctic Fur Seals (*Arctocephalus gazella*) From South Georgia: An Indicator of Long-Term Growth History," *Journal of Zoology* 229, no. 2 (1993): 177–190, https://doi.org/10.1111/j.1469-7998.1993.tb02630.x.

16. V. Hamilton, K. Evans, B. Raymond, and M. A. Hindell, "Environmental Influences on Tooth Growth in Sperm Whales From Southern Australia," *Journal of Experimental Marine Biology and Ecology* 446 (2013): 236–244, https://doi.org/10.1016/j.jembe.2013.05.031.

17. A. C. Myrick, "Examination of Layered Tissues of Odontocetes for Age Determination Using Polarized Light Microscopy," in *Growth of Odontocetes and Sirenians: Problems in Age Determination* (International Whaling Commission, 1980): 105–112, https://swfsc-publications.fisheries.noaa.gov/publications/CR/1980/8063.PDF.

18. R. Stewart and B. Stewart, "Comparison of Between-Tooth Age Estimates of Atlantic Walrus (*Odobenus rosmarus rosmarus*)," *Marine Mammal Science* 21, no. 2 (2005): 346–354.

19. B. A. Armfield, Z. Zheng, S. Bajpai, C. J. Vinyard, and J. Thewissen, "Development and Evolution of the Unique Cetacean Dentition," *PeerJ* 1 (2013): e24, https://doi.org/10.7717/peerj.24.

20. C. A. Hui, "Variability of Dentin Deposits in *Tursiops truncatus*," *Canadian Journal of Fisheries and Aquatic Sciences* 37, no. 4 (1980): 712–716, https://doi.org/10.1139/f80-090.

21. T. Kasuya and S. Matsui, "Age Determination and Growth of the Short-Finned Pilot Whale off the Pacific Coast of Japan," *Scientific Reports of the Whales Research Institute* 35 (1984): 57–91.

22. A. A. Hohn, "Age Estimation," in *Encyclopedia of Marine Mammals* (Academic Press, 2009): 11–17.

23. A. C. Myrick, "Is Tissue Resorption and Replacement in Permanent Teeth of Mammals Caused by Stress-Induced Hypocalcaemia?" in *The Biological Mechanisms of Tooth Eruption and Root Resorption* (EBASCO Media, 1988): 379–389, https://doi.org/10.5555/19892291525.

24. S. D. Newsome, P. L. Koch, M. A. Etnier, and D. Aurioles-Gamboa, "Using Carbon and Nitrogen Isotope Values to Investigate Maternal Strategies in Northeast Pacific Otariids," *Marine Mammal Science* 22, no. 3 (2006): 556–572, https://doi.org/10.1111/j.1748-7692.2006.00043.x.

25. S. Newsome, M. Etnier, D. Monson, and M. Fogel, "Retrospective Characterization of Ontogenetic Shifts in Killer Whale Diets via $\delta^{13}C$ and $\delta^{15}N$ Analysis of Teeth," *Marine Ecology Progress Series* 374 (2009): 229–242, https://doi.org/10.3354/meps07747.

26. N. Hanson, C. Wurster, M. Bird, K. Reid, and I. Boyd, "Intrinsic and Extrinsic Forcing in Life Histories: Patterns of Growth and Stable Isotopes in Male Antarctic Fur Seal Teeth," *Marine Ecology Progress Series* 388 (2009): 263–272, https://doi.org/10.3354/meps08158.

27. T. C. Knox, H. Stuart-Williams, R. M. Warneke, A. J. Hoskins, and J. P. Y. Arnould, "Analysis of Growth and Stable Isotopes in Teeth of Male Australian Fur Seals Reveals Interannual Variability in Prey Resources," *Marine Mammal Science* 30, no. 2 (2014): 763–781, https://doi.org/10.1111/mms.12078.

28. S. Mendes, J. Newton, R. J. Reid, A. Frantzis, and G. J. Pierce, "Stable Isotope Profiles in Sperm Whale Teeth: Variations Between Areas and Sexes," *Journal of the Marine Biological Association* 87, no. 2 (2007): 621–627, https://doi.org/10.1017/S0025315407056019.

29. A. Borrell, A. Velásquez Vacca, A. M. Pinela, et al., "Stable Isotopes Provide Insight Into Population Structure and Segregation in Eastern North Atlantic Sperm Whales," *PLoS ONE* 8, no. 12 (2013): e82398, https://doi.org/10.1371/journal.pone.0082398.

30. L. Stukonytė, A. Borrell, M. Drago, C. Lockyer, G. Víkingsson, and A. Aguilar, "Effect of Formic Acid Treatment on Carbon and Nitrogen Stable Isotope Ratios in Sperm Whale Teeth Dentine," *Rapid Communications in Mass Spectrometry* 37, no. 10 (2023): e9500, https://doi.org/10.1002/rcm.9500.

31. K. R. Bowen and C. M. Kurle, "Reconstructing Foraging Ecology of Predators Using Stable Isotope Analysis of Teeth," Manuscript in preparation (2025).

32. K. Evans and K. Robertson, "A Note on the Preparation of Sperm Whale (*Physeter macrocephalus*) Teeth for Age Determination," *Journal of Cetacean Research and Management* 3, no. 1 (2001): 101–107, https://doi.org/10.47536/jcrm.v3i1.907.

33. C. A. Watt, B. E. Stewart, L. Loseto, T. Halldorson, and S. H. Ferguson, "Estimating Narwhal (*Monodon monoceros*) Age Using Tooth Layers and Aspartic Acid Racemization of Eye Lens Nuclei," *Marine Mammal Science* 36, no. 1 (2020): 103–115, https://doi.org/10.1111/mms.12623.

34. "R: A Language and Environment for Statistical Computing," R Core Team, 2022, https://www.R-project.org/.

35. Posit Team. *RStudio: Integrated Development Environment for R* (2024).

36. M. J. Blanca, R. Alarcón, and J. Arnau, "Non-Normal Data: Is ANOVA Still a Valid Option?," *Psicothema* 29, no. 4 (2017): 552–557, https://doi.org/10.7334/psicothema2016.383.

37. S. H. Ambrose, "Preparation and Characterization of Bone and Tooth Collagen for Isotopic Analysis," *Journal of Archaeological Science* 17, no. 4 (1990): 431–451, https://doi.org/10.1016/0305-4403(90)90007-R.

38. J. A. Lee-Thorp, J. C. Sealy, and N. J. Van Der Merwe, "Stable Carbon Isotope Ratio Differences Between Bone Collagen and Bone Apatite, and Their Relationship to Diet," *Journal of Archaeological Science* 16, no. 6 (1989): 585–599, https://doi.org/10.1016/0305-4403(89)90024-1.

39. E. K. Brault, P. L. Koch, E. Gier, et al., "Effects of Decalcification on Bulk and Compound-Specific Nitrogen and Carbon Isotope Analyses of Dentin," *Rapid Communications in Mass Spectrometry* 28, no. 24 (2014): 2744–2752, https://doi.org/10.1002/rcm.7073.

40. B. Groom, *Effects of Decalcification, Species, and Mammalian Order on Bulk Stable Isotope Values From Marine Mammal Teeth* (San Diego:

University of California, 2018), https://www.proquest.com/openview/7b61f9e80a3bd2171850ad4a04ac42f4/1?pq-origsite=gscholar&cbl=18750.

41. D. M. Post, C. A. Layman, D. A. Arrington, G. Takimoto, J. Quattrochi, and C. G. Montaña, "Getting to the Fat of the Matter: Models, Methods and Assumptions for Dealing With Lipids in Stable Isotope Analyses," *Oecologia* 152, no. 1 (2007): 179–189, https://doi.org/10.1007/s00442-006-0630-x.

42. K. R. Bowen and C. M. Kurle, "Effects of Pre-Treatment, Historical Age, and Sample Characteristics on the Stable Isotope Analyses of Killer Whale (*Orcinus orca*) Bone," *Rapid Communications in Mass Spectrometry* 38, no. 19 (2024): e9874, https://doi.org/10.1002/rcm.9874.

43. R. K. Burton and P. L. Koch, "Isotopic Tracking of Foraging and Long-Distance Migration in Northeastern Pacific Pinnipeds," *Oecologia* 119, no. 4 (1999): 578–585, https://doi.org/10.1007/s004420050822.

44. R. K. Burton, J. J. Snodgrass, D. Gifford-Gonzalez, T. Guilderson, T. Brown, and P. L. Koch, "Holocene Changes in the Ecology of Northern Fur Seals: Insights From Stable Isotopes and Archaeofauna," *Oecologia* 128, no. 1 (2001): 107–115, https://doi.org/10.1007/s004420100631.

45. D. Aurioles, P. L. Koch, and B. J. Le Boeuf, "Differences in Foraging Location of Mexican and California Elephant Seals: Evidence From Stable Isotopes in Pups," *Marine Mammal Science* 22, no. 2 (2006): 326–338, https://doi.org/10.1111/j.1748-7692.2006.00023.x.

46. M. Nishiwaki, "Age Study of Sperm Whale Based on Reading of Tooth Laminations," *Scientific Reports of the Whales Research Institute* 13 (1958): 135.

47. C. H. Lockyer, "A Report on Patterns of Deposition of Dentine and Cement in Teeth of Pilot Whales, Genus *Globicephala*," *Report of the International Whaling Commission* 14 (1993): 137–161.

48. H. Omura, M. Nishiwaki, T. Ichihara, and T. Kasuya, "Osteological Note of a Sperm Whale," *Scientific Reports of the Whales Research Institute* 16 (1962): 35–45.

**Supporting Information**

Additional supporting information can be found online in the Supporting Information section.