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Timing of isotopic integration in marine mammal skull: comparative study between calcified tissues

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RATIONALE: Tissues with different turnover rates have different isotope compositions and reflect the different periods in an animal's life when the isotopes are incorporated during the growth of tissues. Bone is one of the most used tissues for reconstruction of an animal's diet; however, the time of isotope integration remains unknown for many species.
METHODS: The $\delta^{15}N$ and $\delta^{13}C$ values in tooth dentine and bone tissue from the maxilla and mandible of 21 stranded northern elephant seals, *Mirounga angustirostris*, collected on the San Benito and Magdalena Islands, Mexico, between 2000 and 2008 were compared. Bone and dentine samples from each growth layer within the tooth were analyzed using a PDZ Europa ANCA-GSL elemental analyzer interfaced with a PDZ Europa 20-20 continuous flow gas source mass spectrometer.
RESULTS: The $\delta^{15}N$ and $\delta^{13}C$ values were not different between bone structures, indicating similar turnover rates, metabolic activity and amino acid compositions. The differences in the observed $\delta^{13}C$ values between tissues are probably indicative of differences in their amino acid compositions, although the similarities in $\delta^{15}N$ values indicated less variation from different amino acids. Correlation of the analyses between isotopic values of tissues suggests that the maxilla and/or mandible of *M. angustirostris* might reflect the $\delta^{15}N$ signal incorporated during the last 5 years of life of the individuals.
CONCLUSIONS: This study demonstrated the usefulness of the applied approach for providing a best approximation of the timing of isotopic integration into the skull of a marine mammal, thereby reducing uncertainty in exploring historic changes in the species' feeding behavior.

Knowledge of the feeding habits of marine mammals has been advanced using stable isotope analysis (SIA) of nitrogen ($\delta^{15}N$ values) and carbon ($\delta^{13}C$ values).[1,2] This approach is based on the assumption that the isotopic composition of the prey is reflected in the predators' tissues, with enrichment in the heavier isotope at each trophic level.[1,3,4] The tissue-to-diet isotopic fractionation varies with tissue type as a result of differences in the metabolic routing of dietary components between tissues, variation in an animal's growth rate and the nutritional quality of its diet, differences in the protein synthesis, and/or amino acid content.[5,6] SIA of bone tissue has allowed the determination of historic changes in the trophic ecology of species.[7,8] Bone is preserved better than other tissues in natural conditions and, due to its slow turnover rate, it enables the exploration of a large part of the life of an individual.[9,10]

Bone growth is intensive during the early life of an animal (from the fetal phase to early childhood) and slows with age, although it does not stop completely.[11] Continuous re-absorption of old tissue and the deposition of new tissue take place over regular periods throughout life.[12,13] The time of the remodeling process of a bone is different from one portion of the bone to another. For instance, the remodeling time of the trabecular bone is five to ten times faster than that of the cortical bone.[14] Structural differences between bones, such as differences in the density, in the degree of mineralization and mainly variations in the degree of vascularization, may be the key factors regulating this process.[15]

In contrast to bone, dentine is a more appropriate tissue to explore developmental changes in the diet of marine mammals.[6] Teeth grow throughout life, with an annual deposition of dentine layers on the internal surface of the pulp cavity.[11,16,17] Dentine is metabolically inert and is not reabsorbed after deposition. Thus, the first layers deposited, which represent the early life of the individual, are closer to the exterior, and those formed more recently are closer to the pulp cavity (Fig. 1).

In this study, we used SIA of tooth growth layers and bone samples from the maxilla and mandible of northern elephant seals, *Mirounga angustirostris*, to determine differences between tissues and bone structures. Variations are expected due to differences in the biochemical turnover rates of these tissues. The protein synthesis occurs at different speeds according to the tissues' turnover rate and, as a consequence, influences the turnover rate of stable isotopes, reflecting in different time periods of isotopic incorporation into the

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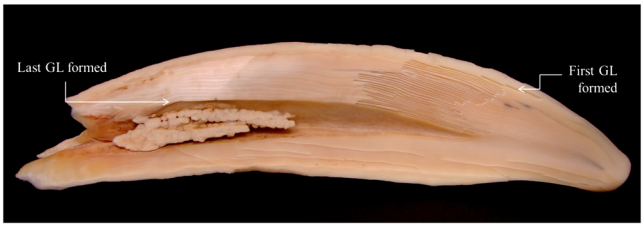

Figure 1. *Mirounga angustirostris*. Longitudinal section of an adult superior canine tooth analyzed. The annual dentine growth layers (GLs) are observed in the upper left side and the marks left by the micro-milling sampling in the upper right side of the tooth. The first layers formed represent the early life of the individual and are closer to the exterior, whereas those formed more recently are closer to the pulp cavity.

tissues.[5,9,18] We explored the relationship between tissues and examined the efficiency of this approach for determining the timing of isotopic integration into the animals' skulls.

This study provides information potentially useful in the field of isotope ecology in the reconstruction of species' diet and in reducing uncertainty in exploring historic changes in their feeding behavior.

EXPERIMENTAL

The $\delta^{15}N$ and $\delta^{13}C$ values from the calcified tissues of stranded animals with known dates of death were determined (Table 1). The teeth and skulls were collected between 2000 and 2008 on the San Benito (28°18.37'N, 115°34.01'W) (8 teeth and 11 skulls) and Magdalena Islands (24°55.45'N, 112°13.50'W) (10 teeth and 10 skulls) on the west coast of Baja California, Mexico.

The age of each individual was estimated by counting dentine growth layers (GLs) in the superior canine tooth. The specimens were distinguished in their age classes:[17,19,20] pups: 28 days, (GL 0); juveniles: 1–4 years, (GL 1–GL 4); subadults: 5–8 years, (GL 5–GL 8); and adults: >8 years, (> GL 8). The teeth were divided longitudinally using a low-speed precision sectioning saw (Isomet-Buehler, Lake Bluff, IL, USA; model no. 11-1280-160) and polished with increasingly finer grades of sandpaper until a smooth finish was achieved.[17,21] Sections were treated with a formic acid solution: 10% for 1 h for juvenile (of 2–4 years) and adult teeth, and 5% for 30 min for the teeth of pups and juveniles of 1 year. The acid etched the surface between the growth layers helping to distinguish them. Because the exposed portion of the tooth was a small fraction of the total sample, it was assumed this portion had no influence on the $\delta^{15}N$ and $\delta^{13}C$ values.[22,23] Dentine samples were taken from each GL using a high-resolution micro-milling system (Merchantek-ESI, Portland, OR, USA) following a sampling path that was ~160 μm wide and ~100–300 μm deep. Pulverized samples were demineralized by repeated treatments with ~0.05 mL 0.1 N HCl for 8–12 h at 6 °C to isolate collagen. Lipids were not extracted from the dentine samples because this tissue does not contain appreciable quantities of ^{13}C-depleted lipids.[16,22]

Bone tissue samples were obtained by perforation with an electric drill in the maxilla and/or mandible of the skull of each individual. Pulverized samples were demineralized by

Table 1. Isotopic signatures from the calcified tissues of the northern elephant seals analyzed. Tooth values are the average of all growth layers (GLs). Age was calculated by counting GLs. The approximate age in some specimens is according to the known date of death and the longitude of the skull. The C/N mass ratio of all samples ranged from 2.8 to 3.6 within the range for unaltered collagen. ID: identification code

Individual ID	Age (years)	$\delta^{15}N$ values (‰)			$\delta^{13}C$ values (‰)			Site and year of collection
		Tooth	Maxilla	Mandible	Tooth	Maxilla	Mandible	
MG1MSd	8	18.6	19.2	18.9	–12.8	–13.0	–13.9	Magdalena-2004
SB4MSd	6	18.9	19.1	17.5	–12.8	–13.8	–16.5	San Benito-2000
SB5F	10	16.9	15.5	15.6	–14.5	–16.3	–15.8	San Benito-2001
SB6M	9	18.3	18.6	18.6	–12.4	–17.1	–15.3	San Benito-2000
MG7J	1	19.9	18.0	17.9	–14.3	–14.1	–14.9	San Benito-2000
SB8J	2	19.4	17.4	16.9	–16.0	–16.8	–15.5	San Benito-2000
MG9J	2	19.1	18.0	17.3	–15.4	–16.3	–15.0	Magdalena-2004
SB10F	8	18.6	18.3	-	–13.8	–16.3	-	San Benito-2008
SB11M	9	17.9	18.3	-	–13.7	–15.7	-	San Benito-2000
SB12M	9	18.2	-	18.1	–13.6	-	–15.7	San Benito-2000
MG29J	1	18.4	-	18.4	–16.9	-	–15.6	Magdalena-2008
MG30J	1	18.6	19.6	18.9	–15.5	–16.3	–15.7	Magdalena-2005
MG31J	1	20.0	20.5	19.6	–14.5	–15.3	–14.9	Magdalena-2004
MG32J	1	19.7	19.2	19.5	–14.1	–15.8	–15.0	Magdalena-2004
MG33J	1	18.8	19.9	19.5	–14.7	–16.1	–15.0	Magdalena-2008
MG34J	2	20.6	19.8	20.0	–14.7	–15.9	–15.4	Magdalena-2005
MG35J	< 2 months	18.7	18.9	19.5	–14.0	–16.1	–15.3	Magdalena-2008
MG36J	1	20.5	20.1	19.9	–14.8	–17.0	–17.3	Magdalena-2005
SB37Sd	> 5	-	17.4	17.3	-	–14.2	–14.5	San Benito-2001
SB38C	< 2 months	-	19.3	19.8	-	–15.1	–16.0	San Benito-2006
SB40C	< 2 months	-	18.0	17.2	-	–16.8	–15.9	San Benito-2000

soaking for 8–12 h in 0.5 N HCl at 6 °C to isolate collagen. Lipids were first extracted using chloroform/methanol/water (2:1:0.8)[24] and then lyophilized.

Isotopic analysis

Dried bone (~0.5 mg) and dentine collagen (~1 mg) were sealed in 8 mm × 5 mm tin capsules and analyzed using a PDZ Europa ANCA-GSL elemental analyzer interfaced with a PDZ Europa 20-20 (Sercon Ltd., Crewe, UK) continuous flow gas source mass spectrometer at the Stable Isotope Facility of the University of California at Davis (Davis, CA, USA). The results are presented in parts per thousand (‰) using the following equation:

$$\delta^{15}N \text{ or } \delta^{13}C = \left(\left(R_{sample}/R_{standard}\right) - 1\right) * 1000‰$$

where R_{sample} and $R_{standard}$ are the values of $^{15}N/^{14}N$ or $^{13}C/^{12}C$ in the sample and standard, respectively. The standards were atmospheric N_2 for nitrogen and Pee Dee Belemnite (PDB) for carbon. The results were calibrated with international standards (ammonium sulfate for $\delta^{15}N$ values and sucrose for $\delta^{13}C$ values), which generated a standard deviation between the isotopic measurement trials of <0.3‰ for $\delta^{15}N$ values and <0.2‰ for $\delta^{13}C$ values. As a control for the quality of the dentine and bone collagen, the carbon to nitrogen (C/N) mass ratio of all samples was measured. The ratios ranged from 2.8 to 3.6, well within the range for unaltered collagen.[4]

Data analysis

The individuals were discriminated in two age groups, juveniles (up to 4 years) and adults (≥ 5 years). Isotopic differences between both sections of the skull and the teeth from juveniles and adults were assessed using a two-way analysis of variance (ANOVA) test along with a Tukey test for multiple comparisons. The relationship between bone collagen isotopic values from the mandible and maxilla of 17 individuals was examined by correlation analysis. The isotope timing integration in the skull of the animals was explored by the relationship between the bone and tooth collagen of 18 individuals with both tissues. For this, a series of simple correlation exercises was performed between the bone value and the average value obtained from the sum of different GLs within the tooth of each individual. The tooth mean values were calculated when all the GLs were summed, then when the outer layer (i.e., the first formed) was not included in the sum, and so on successively. The calculations were performed until the innermost GLs were excluded according to the age of the individuals. The results were arranged in a matrix from the last to the first GL formed (Table 2).

The correlation analyses were performed between the values of bone and (a) the average of all the GLs within the tooth, (b) the value of the last GL formed (yearlings were not included), (c) the mean value of the last two GLs, (d) the last three GLs, (e) the last four GLs, (f) the last five GLs (individuals of 1 and 2 years old were not included in these last four analyses), (g) the last six GLs (individuals under 6 years old were not included), (h) the last seven GLs (individuals under 7 years old were not included), and (i) the last eight GLs (individuals under 8 years old were not included). The highest correlation value calculated determined the most accurate time of isotopic integration in the animals' skull. Statistical analyses were performed using the software Statistica 8.0 (StatSoft. Inc., Tulsa, OK, USA). Statistical significance was assumed at $P < 0.05$.

RESULTS

The isotope values from the bony structures and the teeth of 21 animals were compared. No statistically significant differences in $\delta^{15}N$ values among the structures or per age class were found (two-way ANOVA: $F_{(2,50)} = 0.93$, $P = 0.399$ and $F_{(2,50)} = 0.11$, $P = 0.896$, respectively). For the $\delta^{13}C$ values, the differences were significant among the structures (two-way ANOVA: $F_{(2,50)} = 13.16$, $P < 0.001$), with the tooth different from the maxilla and the mandible (Tukey: $P = 0.0003$ and $P = 0.002$, respectively). The differences in the $\delta^{13}C$ values were due to the individuals' age class (two-way ANOVA: $F_{(2,50)} = 3.55$, $P = 0.036$). $\delta^{13}C$ values from the teeth were significantly different between juveniles and adults (Tukey: $P = 0.010$), but the $\delta^{13}C$ values from the maxilla and the mandible were not significantly different between age classes (all Tukey: $P > 0.05$).

The $\delta^{15}N$ values from the maxilla and the mandible of the same skull in 17 individuals were highly correlated (Pearson coefficient correlation: $r = 0.90$, $P < 0.001$) (Fig. 2), and, for the $\delta^{13}C$ values, the correlation was lower but still significant ($r = 0.48$, $P < 0.05$). For the correlation analyses between tissues, the isotope values from the maxilla (in the majority of the individuals) or mandible (from individuals without maxilla samples) were used because there were not significant differences between the structures. For the $\delta^{13}C$ values, there was not a significant correlation between tissues in any case compared (all $P > 0.05$) (Table 3). For $\delta^{15}N$ values, the highest correlation was between the bone and the average value of the last five GLs formed ($r = 0.94$, $P < 0.01$). This finding indicated that the bone tissue might reflect the $\delta^{15}N$ signal incorporated for approximately the last five years in the life of this seal species.

DISCUSSION

The bone collagen $\delta^{15}N$ and $\delta^{13}C$ values of mammals reflect the mean $\delta^{15}N$ and $\delta^{13}C$ values of all proteins consumed by an individual during its last several years of life.[10] Different bone remodeling rates might cause different isotopic compositions among various bones from the same individual, due to the varying rates of bone turnover among the different skeletal elements. The fraction of bone remodeled per year defines a turnover rate which change with age.[25] Structural differences between bones, mainly variations in the degree of vascularization, might be the key factor regulating the remodeling process.[15] Highly mineralized bones such as mandible have slower remodeling and turnover rates than post-cranial bones,[15] thereby reflecting different periods of isotopic integration among distinct skeletal bones.

However, the secondary effects of isotope fractionation also determine the assimilation of nitrogen and carbon in tissues or in different portions of the same tissue, mainly because of

Table 2. *Mirounga angustirostris*. Values of $\delta^{15}N$ and $\delta^{13}C$ (in brackets) in the bone and tooth collagen of the 18 individuals analyzed. The tooth collagen (TC) values are the average of the sum of different dentine growth layers (GLs) within the tooth of each individual. The TC mean values were calculated when all the GLs were summed, then when the outer layer (i.e., the first layer formed within the tooth) was not included in the sum, and so on successively. The calculations were performed until the innermost GLs were excluded according to the age of each individual. The TC mean values were arranged from the last to the first growth layer formed in the tooth and are shown in that order.ID: identification code

	$\delta^{15}N$ & $\delta^{13}C$ values (‰)	Tooth collagen								
Individual ID	Bone collagen	All GLs	Last GL	Last 2 GLs	Last 3 GLs	Last 4 GLs	Last 5 GLs	Last 6 GLs	Last 7 GLs	Last 8 GLs
SB5F	15.55 (−16.34)	16.88 (−14.49)	15.25 (−15.02)	15.38 (−14.88)	15.86 (−14.63)	16.16 (−14.53)	16.48 (−14.50)	16.47 (−14.76)	16.47 (−14.70)	16.59 (−14.63)
SB6M	18.61 (−17.05)	18.29 (−12.41)	19.79 (−12.55)	19.07 (−12.43)	18.58 (−12.43)	18.56 (−12.39)	18.52 (−12.41)	18.49 (−12.38)	18.40 (−12.37)	18.32 (−12.39)
SB11M	18.28 (−15.68)	17.86 (−13.73)	16.39 (−13.81)	16.88 (−13.89)	17.14 (−13.90)	17.37 (−13.91)	17.74 (−13.94)	17.59 (−13.91)	17.48 (−13.86)	17.55 (−13.78)
SB12M	18.09 (−15.74)	18.17 (−13.60)	18.68 (−14.44)	18.56 (−14.04)	18.40 (−13.99)	18.17 (−13.90)	18.25 (−13.85)	18.22 (−13.81)	18.07 (−13.75)	18.06 (−13.71)
MG1MSd	19.15 (−12.95)	18.57 (−12.84)	16.76 (−14.08)	17.82 (−13.57)	18.22 (−13.34)	18.34 (−13.16)	18.38 (−13.00)	18.33 (−12.93)	18.43 (−12.90)	
SB10F	18.31 (−16.34)	18.63 (−13.77)	18.39 (−13.33)	18.25 (−13.59)	18.19 (−13.70)	18.27 (−13.80)	18.29 (−13.76)	18.31 (−13.73)	18.36 (−13.71)	
SB4MSd	19.07 (−13.84)	18.91 (−12.81)	18.79 (−12.87)	18.80 (−12.72)	18.75 (−12.63)	18.79 (−12.62)	18.81 (−12.58)			
SB8J	17.42 (−16.84)	19.38 (−16.02)	18.51 (−14.69)							
MG9J	18.03 (−16.31)	19.10 (−15.35)	18.48 (−13.92)							
MG33J	19.86 (−16.08)	18.81 (−14.68)	19.54 (−14.88)							
MG34J	19.78 (−15.86)	20.58 (−14.65)								
MG35J	18.85 (−16.07)	18.70 (−13.98)								
MG36J	20.14 (−17.04)	20.54 (−14.78)								
MG29J	18.42 (−15.60)	18.42 (−16.87)								
MG30J	19.64 (−16.27)	18.55 (−15.47)								
MG31J	20.48 (−15.32)	20.04 (−14.50)								
MG32J	19.17 (−15.79)	19.75 (−14.10)								
SB7J	18.04 (−14.05)	19.89 (−14.31)								

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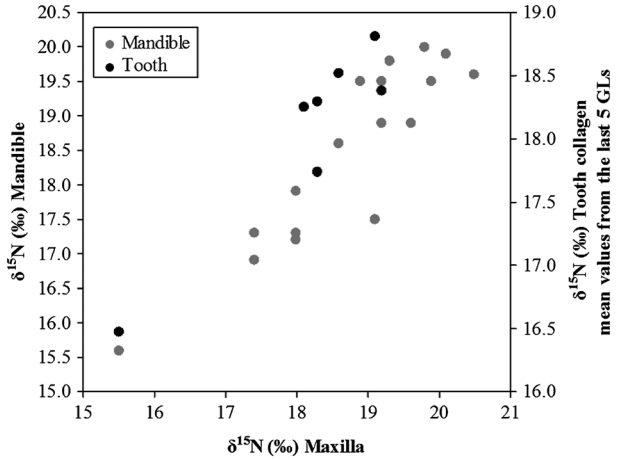

Figure 2. *Mirounga angustirostris*. $\delta^{15}N$ correlation plot between the bone collagen values from the maxilla (*x*-axis) and mandible (*y*-left axis), as well as between the maxilla and the tooth collagen (*y*-right axis) mean values of the last five growth layers (GLs) formed within the tooth of the specimens.

differences in metabolic activity but also to differences in the synthesis of proteins and/or the amino acid composition.[5] We did not find significant differences between the maxilla and the mandible isotopic values, indicating that these structures have similar turnover rates, metabolic activity and/or amino acid composition.

Tissues with different turnover rates have different isotope compositions.[13] There is an apparent correlation between the turnover rate and the specific metabolic activity of tissues.[18,26] Furthermore, individual proteins within each tissue have specific turnover rates depending on the metabolic functions performed.[26] Differences in amino acid content and isotope composition can account for much of the variation between different proteins.[13] Tissues with similar amino acid contents should display a similar isotopic composition, unless the same amino acid has different isotopic values in each protein.[27]

Both dentine and bone have inorganic and organic components. The organic phase of dentine is 90% collagen fibrils (mainly type I with small amounts of type III and V) with fractional inclusions of various non-collagenous matrix proteins (NCPs) and lipids.[11,28] The NCPs fall into several categories such as phospho-proteins, Gla-proteins of the osteocalcin type, as well as matrix Gla-proteins, proteoglycans, different acidic glyco-proteins, and serum proteins.[29] Bone organic matter is mainly formed by collagen type I, which constitutes 90% to 95% of the total organic matrix, as well as other non-collagenous proteins[11,28] such as osteocalcin, osteonectin, osteopontin, and lipids.[30] Although studies have for a long time focused on identifying proteins specific to bone or dentine, it is now clear that bone matrix proteins can be found in dentine and that dentine matrix proteins also are present in bone.[28]

Bone collagen is unusual in containing high proportions of the amino acids, glycine (33% of total amino acids) and hydroxypoline (9%). Individual amino acids from collagen or muscle can differ greatly in their $\delta^{13}C$ values.[31,32] The distinctive amino acid composition of bone collagen is recognized to produce larger than normal diet-tissue ^{13}C fractionation.[6] While 'soft' tissues such as muscle, liver, and skin are ^{13}C-enriched by only 1–2‰ relative to diet, bone collagen typically has $\delta^{13}C$ values that are 4–5‰ higher than those of the diet.[33]

Amino acid composition differences between dentine and bone tissue provide an adequate explanation for the observed differences in $\delta^{13}C$ values. In contrast studies have demonstrated that in a single tissue there is less variation in the $\delta^{15}N$ isotopic values from different amino acids,[27,31] which might explain the similarities found among tissues in our study. This finding demonstrates that the nitrogen isotope is more widely affected by metabolic processes than the carbon isotope, as suggested by other authors,[1,2,27] indicating that the nitrogen isotope is a better trophic indicator.

The protein turnover rates influence the isotope turnover rates, which, in turn, reflect the time period of isotope incorporation into the tissues.[5,9,18] Dentine is a metabolically inactive tissue that reflects isotopes incorporated during its growth. Thus, each layer formed within the tooth represents isotopes incorporated in each year of the life of an animal. Bone experiences a turnover, thus representing the time when it was formed in the animal's lifetime, depending on the animal's age or type of bone.[13] We did not find significant isotope differences in the maxilla or in the mandible between juveniles and adults, although such differences were expected due to the more intensive growth during early life than during adulthood.[11,14]

Table 3. Correlation analyses (r values) between the bone and tooth collagen isotopic ($\delta^{15}N$ and $\delta^{13}C$) values of the northern elephant seals analyzed. The significant values ($P < 0.05$) are presented in bold. See Table 2 for the data that were employed in the analyses. BC: bone collagen, TC: tooth collagen

BC vs. TC	No. of related samples	$\delta^{15}N$	$\delta^{13}C$
BC vs. TC mean value from all GLs	36	**0.65**	0.35
BC vs. TC value of the last GL	20	0.62	0.13
BC vs. TC mean value of the last 2 GLs	14	**0.82**	0.13
BC vs. TC mean value of the last 3 GLs	14	**0.88**	0.22
BC vs. TC mean value of the last 4 GLs	14	**0.92**	0.27
BC vs. TC mean value of the last 5 GLs	14	**0.94**	0.33
BC vs. TC mean value of the last 6 GLs	12	**0.92**	0.17
BC vs. TC mean value of the last 7 GLs	12	**0.92**	0.18
BC vs. TC mean value of the last 8 GLs	8	**0.93**	-0.58

In pinnipeds such as *Callorhinus ursinus* and *Zalophus californianus*, the bone turnover in young-of-the-year was suggested to occur in ~8–10 months and ~10–12 months, respectively.[23] The isotope values in individuals of 1 year were higher than those of adults and related to the nursing effect, which determine an enrichment in ^{15}N and a depletion in ^{13}C between mother and offspring.[21–23]

The northern elephant seal feeds exclusively on its mother's milk in the first 26 to 28 days of age and then takes up its independent feeding as a juvenile.[34] If the bone turnover in yearling northern elephant seals is approximated to that suggested for *C. ursinus* and *Z. californianus*, this might explain the higher $\delta^{15}N$ values in juveniles of 1 year than in adults found in our study. The isotope values reflect the enrichment in ^{15}N because of the lactation effect (during the pup phase), but slightly diminished owing to the inclusion of prey of low trophic level consumed during the first months as juvenile.

However, latitude differences in the feeding sites between juveniles and adults might be the principal cause for the observed differences in $\delta^{15}N$ and $\delta^{13}C$ values, although they are statistically indistinguishable.[17] Moreover, the differences in the $\delta^{13}C$ values in dentine between juvenile and adult elephant seals might correspond to their distinct feeding strategies.[17]

Based on the correlation analyses between the $\delta^{15}N$ values of bone and dentine from different growth layers, we hypothesize that the bone collagen from the maxilla and/or mandible of northern elephant seals integrates the $\delta^{15}N$ values for approximately the last five years of life of the individuals. However, analysis of a more extensive sample is needed to strengthen this hypothesis.

CONCLUSIONS

This study demonstrated the usefulness of the applied approach for providing a best approximation of the timing of isotopic integration into the skull of a marine mammal, the northern elephant seal *Mirounga angustirostris*, thereby reducing uncertainty in exploring historic changes in the feeding behavior of the species. This approach is not restricted to this species and may also be applied to other species taking into account the considerations discussed above.

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