Are stable isotope ratios and oscillations consistent in all baleen plates along the filtering apparatus? Validation of an increasingly-used methodology

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ABSTRACT

RATIONALE: Baleen plates are anatomical structures composed of inert tissue that hang from the upper jaw in mysticetes. Baleen plates may differ in size and in coloration between different segments of the filtering row or between sides of the mouth. Concern has been raised that variation in baleen plate characteristics may reflect dissimilar structural composition and growth rates liable of affecting the stable isotope ratios and their oscillation patterns.

METHODS: We measured stable carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) isotope ratios along the longitudinal axis of six baleen plates collected from different positions along the mouth of a fin whale. All samples were analyzed in a continuous flow isotope ratio mass spectrometer.

RESULTS: A total of 206 samples were analyzed. When comparing the first 18 cm of each baleen plate, Kruskal-Wallis test showed no significant differences neither for $\delta^{13}C$ ($p = 0.08$) nor for $\delta^{15}N$ ($p = 0.58$) values. Visually, all baleen plates presented nearly identical oscillations, independently of the position or the coloration of the baleen plate.

CONCLUSIONS: Differences in size between plates in an individual are due to differential erosion rates according to their position in the mouth. Therefore, position of sampling along the baleen plate row should not be a reason of concern when conducting stable isotope studies.

Keywords: Fin whale, mysticete, nitrogen, carbon, $\delta^{15}N$, $\delta^{13}C$

INTRODUCTION

In the last decades, stable isotope analysis has become a standard tool in animal ecology studies, particularly to investigate diet composition, migration and physiology of individuals in the wild [1, 2]. In marine mammals, this technique has experienced substantial development [3] because these animals are difficult to observe or handle, and therefore many of their biological traits can only be determined through the application of chemical markers. The stable isotope ratios of nitrogen ($^{15}N/^{14}N$, expressed as $\delta^{15}N$) and carbon (^{13}C / ^{12}C, expressed as $\delta^{13}C$) are markers commonly used because they inform about diet, trophic level and the characteristics of the ecosystem in which the animal feeds [4-7].

Stable isotope studies can be carried out on any body tissue, although each tissue has different discrimination factors and turnover rates [8-10]. Some bones, otoliths and teeth, as well as keratinous tissues such as feathers, hair, nails or baleen plates are biologically inert, which means that their biogeochemical composition does not vary after the tissue is consolidated. In the cases in which such tissues experience continuous growth, a chronologically-sequential record of the environment in which the animal has lived is preserved in successive growth layers. This property has been used to infer variations in physiology or habitat use during periods of the life cycle of individuals that otherwise would be impossible to monitor [11-15].

Baleen plates grow continuously and therefore they sequentially archive the stable isotopic seascape of the water mass in which the whale lives or its variation in feeding regimes [16-18]. Schell et al. were the first to measure stable isotopes along the growth axis of a baleen plate, obtaining a temporal record of recent movements and

diet [19]. Since then, many studies have followed this approach to gain information on migration and diet shifts in a variety of baleen whale species [17, 20-23]. Because many whale species or populations stay during part of their life cycle in unknown geographical destinations, baleen plates provide an invaluable insight into these periods that otherwise would remain obscure.

Baleen plates hang from the upper jaw in bilateral rows along the rostrum and, depending on their position, they greatly vary in size; those in the central-posterior region are the largest baleen, with sizes diminishing caudally and distally [24]. Besides, the color of the plates varies between species but, more importantly, in some baleen whales the color of the baleen plates may vary between different segments of the row or between sides of the mouth. The heterogeneity in size according to position in the mouth may be due to differences in the baleen plate growth rates. If this were the case, the amplitude of the oscillations of the stable isotope ratios along the baleen plate would differ between plates of different size. In addition, differences in coloration may reflect dissimilar structural composition, also potentially affecting the isotope ratios. Concern about these issues has been expressed in some previous studies but never addressed through specifically-designed experiments, thus remaining unresolved [19, 25-28].

With the aim of optimizing the use of baleen plates to investigate the ecology of mysticetes, we investigate here the potential effect that non-standardized sampling of baleen plates may have on stable isotope ratios and their oscillations along the plates. We have examined the replicability of stable isotope patterns between the baleen plates of a same fin whale, but occupying different positions in the mouth and thus having dissimilar size and coloration. The fin whale was selected because, as

most baleen whales, it undertakes annual migrations alternating high-latitude summer grounds with low-latitude winter-grounds [29] and clear oscillations of the stable isotope ratios have been observed in their baleen plates [17, 27, 30]. In addition, the fin whale is the mysticete in which the coloration of baleen plates shows the highest heterogeneity and asymmetry [29], thus permitting to test for the potential effect of bilaterality or coloration-related differences.

MATERIALS AND METHODS

Sample collection and preparation

The baleen plates were obtained from a 17.40 m male fin whale flensed at the Hvalur H/F whaling station (Hvalfjordur, Iceland) on 8 August, 2015. The length of the baleen filtering apparatus on the right side of the mouth was measured and five plates were collected in roughly equidistant positions from the tip identified as A, B, C, D and E (see Fig. 1). An additional plate, identified as O, was collected from the left maxilla in the position equivalent to position C.

The baleen plates were labelled and initially preserved at -20ºC. Once at the laboratory, they were thawed, the gum was removed with steel wool to allow adequate sampling of the keratin plate, and the surface of the plate was cleaned for external or adhered materials using steel wool and a chloroform:methanol solution (2:1). Once clean, the plates were stored dry until analysis. The subsamples used for the stable isotope analysis were extracted with a grinder delineating parallel rows separated by 1 cm and starting from the proximal part of the baleen (that most recently formed) to the most distal (the oldest part of the plate). The number of subsamples varied between plates according to their length.

Stable Isotopes Analysis

Approximately 0.3 mg of the powered subsamples were weighed into tin capsules. Samples were automatically loaded and combusted at 1000ºC to be analyzed in a continuous flow isotope ratio mass spectrometer (ThermoFinnigan Flash 1112 elemental analyzer; CE Elantech, Lakewood, NJ, USA), coupled to a Delta C isotope ratio mass spectrometer via a ConFlo III interface (both from ThermoFinnigan, Bremen, Germany). International isotope secondary standards of known $^{13}C/^{12}C$ and $^{15}N/^{14}N$ ratios, namely: polyethylene (IAEA CH7; $\delta^{13}C = -31.8‰$), sucrose (IAEA CH6; $\delta^{13}C = -10.4‰$), ammonium sulphate (IAEA N1; $\delta^{15}N = +0.4‰$ and IAEA N2; $\delta^{15}N = +20.3‰$), potassium nitrate (USGS 34; $\delta^{15}N = -1{,}7‰$), L-glutamic acid (USGS 40; $\delta^{15}N = -4{,}6$ ‰; $\delta^{13}C = -26{,}2‰$), and caffeine (IAEA 600; $\delta^{15}N = 1{,}0‰$; $\delta^{13}C = -27{,}7‰$) were used to calibrate the system and compensate for any analytical drift over time. The reference materials used for the analysis were selected according to previous calibration experiments performed on the same type of samples to ensure that the range of the reference values spanned those of the samples.

Stable isotopes ratios are expressed following the delta (δ) notation, while the relative variations of stable isotope ratios are expressed as per mil (‰) deviations from the predefined international standards according to the equation:

$$\delta X = (R_{sample}/R_{standard}) - 1$$

where X is ^{13}C or ^{15}N, and R_{sample} and $R_{standard}$ are the heavy-to-light isotope ratios ($^{13}C/^{12}C$ and $^{15}N/^{14}N$) in the sample and in the reference standards, respectively. . These standards are the Vienna Pee Dee Belemnite (V-PDB) calcium carbonate for ^{13}C and the atmospheric nitrogen (air) for ^{15}N. The precision and accuracy for $\delta^{13}C$ and $\delta^{15}N$ measurements were 0.1‰ and 0.3‰, respectively. These analyses were

conducted in the “Centres Cientifics i Tecnològics” of the University of Barcelona (CCiT-UB).

Data Analysis

With the aim of visually comparing oscillations between the baleen plates, isotopic ratios of carbon and nitrogen were individually examined by fitting a generalized additive model (GAM) to the data from each baleen plate using mgcv package [31] in R [32]. Each model was fitted considering the isotope ratios of each element as the dependent variable and the length of the different baleen plates as the independent variable. For each baleen plate and isotope ratio, homoscedasticity and normality of the residuals were checked, and models were adjusted by removing outliers and choosing best k. All the parameters are specified in the Table S1.

$\delta^{15}N$ and $\delta^{13}C$ values were examined in the first 18 cm (starting from the gum) of each plate, which roughly included the most recent complete migratory cycle of the whale [17]. Data were tested for normality (Shapiro-Wilk test) and homoscedasticity (Levene’s test), and means and standard deviations were calculated for each baleen plate. To investigate whether the variability between plates was constant along all the plate length, standard deviation values in each data point were calculated and plotted (Figure S1). Finally, differences between the means of the baleen plates were analysed through a Kruskal-Wallis test.

RESULTS

A total of 206 samples were analysed. Pigmentation and number of points analysed for each baleen plate, as well as mean and standard deviations of $\delta^{15}N$ and $\delta^{13}C$

values in the first 18 cm of each baleen plate, are detailed in Table 1. Standard deviations for each data point along the first 18 cm of baleen plate length are shown in FigS1. Almost all data points had standard deviations around or below 0.3, in agreement with the analytical precision of the $\delta^{13}C$ and $\delta^{15}N$ measurements (see Materials and Methods). Despite this general trend, some segments of the baleen plates showed higher standard deviations. For $\delta^{15}N$ values this occurred in the first 3 data points situated in the proximal part of the baleen plate, and for $\delta^{13}C$ values this occurred in the data points 14 and 15. In both cases these points coincide with the segments of the baleen plates where the $\delta^{15}N$ and $\delta^{13}C$ values undergo a rapid change (Fig 2).

All baleen plates showed oscillations in their $\delta^{13}C$ and $\delta^{15}N$ values along their growing axis (Fig. 2), and trends were nearly identical in all baleen plates. The Kruskal-Wallis test showed no significant differences neither for $\delta^{13}C$ ($p = 0.08$) nor for $\delta^{15}N$ ($p = 0.58$), when comparing the first 18 cm of each baleen plate.

DISCUSSION

Validation and standardization of the sampling of archival tissues to infer ecological and physiological traits, variation in diet, or migration have been conducted in a number of species and for a variety of keratinous structures, such as human and other animal hair, pinniped vibrissae or bird feathers [11, 33-35]. These studies have involved assessment of variability within individuals and within repeated samples of the same individual. However, possibly because of the difficulty of acquiring adequate samples, baleen plates have not so far been subject to extensive methodolog-

ical studies despite expressed concerns about the potential non-replicability between baleen plates from the same individual.

Schell et al. [19] and Lubetkin et al. [26] investigated oscillations between two opposite plates in a bowhead whale (*Balaena mysticetus*), and Caraveo-Patiño and Soto [25] compared two consecutive plates in a grey whale (*Eschrichtius robustus*). In all cases the oscillations found in the various plates were very similar, although the plates selected had in all cases been obtained from approximately the central-posterior part of the filtering apparatus, where the size of the plates is larger. As a consequence, the potential effect of differential growth rates according to plate size or position in the maxilla, if occurring, could not be appropriately tested. Only two studies, that of Eisenmann et al. [28] in humpback whales (*Megaptera novaeangliae*) and that of Bentaleb et al. [27] in fin whales have compared baleen pairs of plates of different size belonging to the same individual, obtaining in the two cases dissimilar results: the first found nearly identical patterns in each pair of plates, while the second found different oscillations between the plates although the mean values of $\delta^{15}N$ and $\delta^{13}C$ for corresponding segments of the plates were found to be similar.

With the aim of clarifying this issue, we analyzed 6 baleen plates collected from different positions in the mouth of the same animal. The highest variability between plates was found in segments of the baleen plates in which the change in the stable isotope ratios occurs fast (FigS1, Fig2). Due to such rapid modification in the stable isotope ratios, small variations in determining the sampling location likely produce large differences in the stable isotope ratios results. To overcome this, we suggest that the segments of the baleen plates subject to faster changes in stable isotope

ratios should be sampled at smaller intervals (for example a few milimetres apart) than the rest of segments.

Nonetheless, the stable isotope ratios observed throughout the baleen plates of different sizes, and sampled in different positions along the filtering apparatus, presented nearly identical oscillations (Fig. 2). In addition, results from Kruskal-Wallis test, performed with the stable isotope ratios in the first 18 cm of each plate showed no significant differences among plates. This similarity among isotopic patterns and means point out that all baleen plates grow at similar rates and that differences in plate size are due to the differential erosion at which the plate is subjected according to their position in the mouth, as it has been historically assumed [36, 37]. Records of variation in thickness in different baleen plates proceeding from a single animal suggest that short plates present the same pattern as the long plates' upper part. Thus, shorter baleen plates seem to be exposed to a greater erosion than longer plates [38]. However, until now this hypothesis had only been confirmed in grey whales [39, 40].

Another potential source of heterogeneity in sampling between plates is coloration. A number of mysticetes show some degree of asymmetry in body pigmentation, and different segments of the filtering apparatus may show dissimilar plate coloring. In the fin whale (*Balaenoptera physalus)* the asymmetry is extremely marked: in the left side, the lower jaw is dark grey and the plates are gray, while in the right side the lower jaw is white and the rear two-thirds of the plates are gray but those on the front third are yellowish [29]. In the sei whale (*Balaenoptera borealis*), most baleen plates are dark gray but those in the front tend to be whitish [41]. In the dwarf minke whale, *Balaenoptera acutorostrata* [42] and in Omura's whale, *Balaenoptera omurai*, baleen

plates do not show marked asymmetry but the coloration of the head does, although in the latter species the asymmetry is reversed as compared to the fin whale: the lower jaw area is black on the left side and white on the right [43]. The reasons for the differences in coloration and size of the plates are unclear, but it is generally accepted that they reflect dissimilarities in function between mouth segments or sides [44].

The potential effect of pigmentation on the stable isotope ratios has been investigated in the skin of different species of cetaceans. Thus, values in the dorsal region of the body trunk (typically dark-colored) has been compared with the ventral region (typically white or pale-colored) in striped (*Stenella coeruleoalba*) and common dolphins (*Delphinus delphis*) [45], bottlenose dolphins (*Tursiops truncatus*) and killer whales (*Orcinus orca*) [46], and in all cases resulting values were statistically undistinguishable. Also, studies in human hair have shown that loss of pigment has no effect on the C/N, $\delta^{15}N$ and $\delta^{13}C$ values [47, 48], all indicating that coloration *per se* should not be expected to have any effect on stable isotope ratios.

The asymmetrical coloration of both the rostral region and the baleen plates that occurs in some mysticetes is commonly thought to serve in the maintenance of the counter shading when the whale rolls to its side during feeding lunges, or to aid in startling prey and elicit its aggregation [49, 50]. However, this hypothesis does not appear to be clearly supported by field data [44]. If the asymmetrical variation is limited to pigmentation, the above evidences from skin and hair would point to a non-effect on stable isotope ratios of baleen plates of different coloration. However, it can be reasonably argued that the evolutionary forces that have brought different segments of the baleen plate rows, or of different sides of the mouth, to acquire dissimi-

lar colorations may also reflect differences in function of the filtering apparatus and therefore may have also affected the mechanical properties of the baleen and their structure, rate of growth or rate of erosion. Thus, tendency to selectively roll to one side or another during feeding may induce differential mechanical tensions or differential erosion to the plates in each body side. Independently of whether this is true or not, the results of the present study shows that the stable isotope ratios and their oscillation patterns were statistically indistinguishable either between plates displaying contrasting coloration or between plates sampled in the same position of the filtering row but collected from opposite sides of the mouth.

We can conclude from the above that all baleen plates, independently of their position in the filtering apparatus, size or coloration, grow at the same rate and display similar stable isotope ratios and oscillations. Differences in size between plates in a same individual are thus solely due to differential erosion rates depending on the position of the baleen plates in the mouth. Therefore, position of sampling along the baleen plate row should not be a significant source of concern with regards to sampling for stable isotope studies. However, in the segments where stable isotope ratios change rapidly, it would be recommendable to sample at smaller intervals than in the other segments to obtain a precise trend of the isotopic ratios along the whole plate. In addition, with the aim of optimizing and standardizing procedures, it is recommended that whenever possible baleen plates should be sampled in the central position of the left row, which in most species is dark-colored and are among the largest in the filtering apparatus, thus providing the longest time span for investigating seasonal oscillations.

ACKNOWLEDGEMENTS

Samples were provided by the Marine Research Institute of Iceland (Hafrannsóknastofnun). Authors are grateful to the personnel of the Marine Research Institute who conducted the fieldwork, as well as to the staff of the Centres Científics i Tecnològics (CCiT-UB) of the University of Barcelona, where the stable isotope ratio determinations were carried out. This work was supported by project CGL2015-70468-R (MINECO/FEDER, UE).

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Table 1: Characteristics of the plates analyzed in this study and $\delta^{15}N$ and $\delta^{13}C$ values (mean ± s.d., permil) for the first 18 cm.

Plate	Pigmentation	Samples	$\delta^{15}N$ ± s.d.	$\delta^{13}C$ ± s.d.
A	Yellowish	20	9.7 ± 0.7	-18.4 ± 0.5
B	Yellowish	29	9.8 ± 0.5	-18.7 ± 0.5
C	Grey	45	9.8 ± 0.5	-18.8 ± 0.5
D	Grey	34	9.8 ± 0.4	-18.9 ± 0.5
E	Slate Grey	34	10.1 ± 0.4	-18.7 ± 0.5
O	Grey	44	9.9 ± 0.4	-18.7 ± 0.5

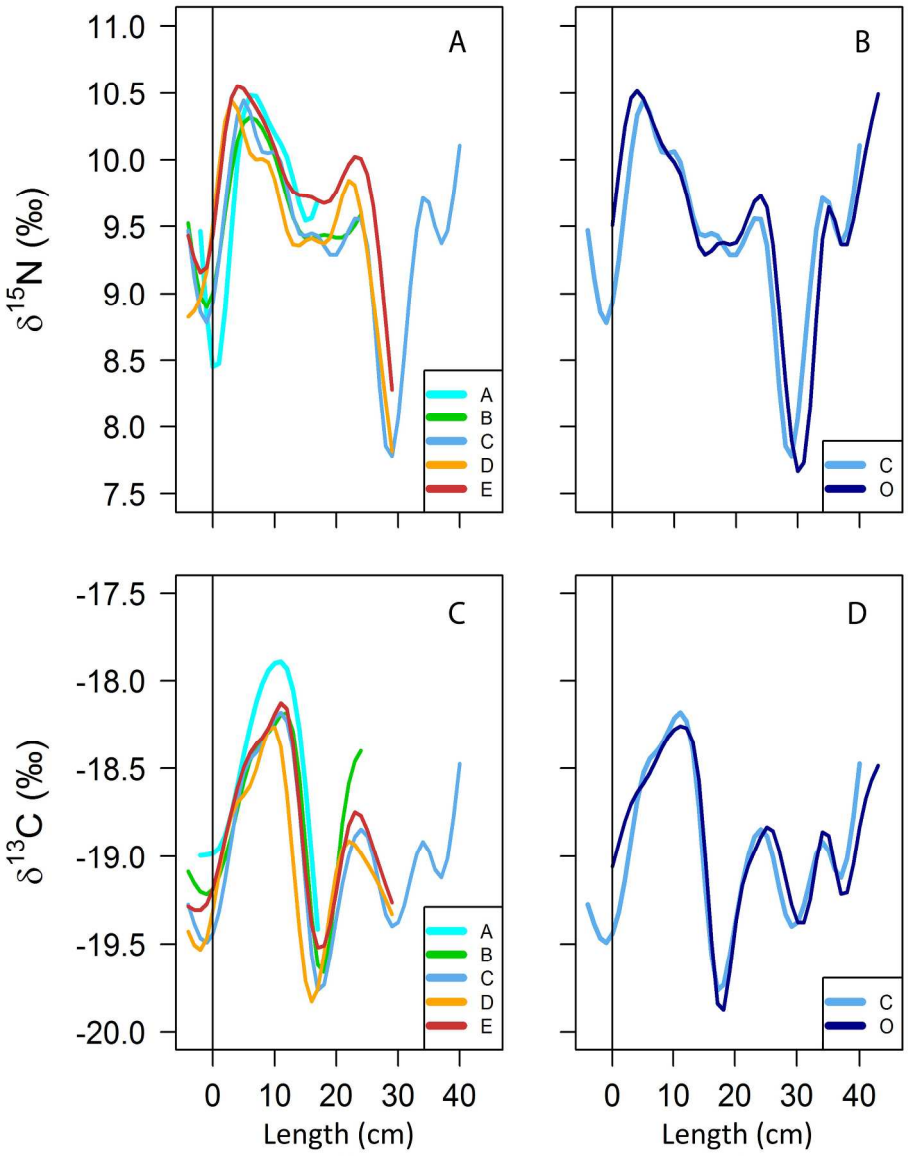

Fig. 2. Oscillations of δ15N (A and B panels) and δ13C values (C and D panels) along the growing axis of the various plates from the left row (A and C panels), and comparison of plates occupying central positions in each body side: C in the left side and O in the right (B and D the panels).

236x308mm (300 x 300 DPI)

Fig. 1. Place of sampling of each plate. The total length of the filtering apparatus was 362cm. The five plates from the right maxilla were collected from roughly equidistant positions from the tip: A at 45cm, B at 90cm, C at 180cm, D at 270cm, and E at 316cm. Plate O was collected from the left maxilla at the position equivalent to position C.

74x30mm (300 x 300 DPI)