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

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ABSTRACT

Baleen plates are composed of inert tissue that grows incrementally and that therefore archives in a sequential manner the stable isotopic values of the whale body pool during a time span of several years. Baleen plates differ in size, and in some species also 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 values and their oscillation patterns. Here we investigate in a fin whale the potential effect that non-standardized sampling of baleen plates may have on the stable isotope results by examining the replicability of patterns between baleen plates occupying different positions in the mouth and thus having dissimilar size and coloration. Results show 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 values and oscillations. We conclude that 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. However, with the aim of optimizing and standardizing procedures, it is recommended that plates are sampled in the central position of the left row.

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 (Hobson 1999; Kelly 2000). In marine mammals, this technique has experienced substantial development (Newsome et al. 210) because these animals are difficult to observe, capture or handle, and therefore many of their biological traits can only be determined through the application of chemical markers or other similarly indirect techniques. Among the various elements that have deserved the attention of ecologists, 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 those most commonly used because they inform about diet, trophic level and the characteristics of the ecosystem in which the animal feeds (DeNiro and Epstein 1981; Peterson and Fry 1987; Fry 1988; Hobson et al. 1994).

Stable isotope studies can be carried out on any body tissue, although each tissue has a different discrimination factor, this is, the variation between the mean stable isotope value of the ingested

food and that of the tissue (Caut et al. 2009; Borrell et al. 2012). Also, each tissue has a different turnover rate, this is, the time required for a tissue to change its isotopic composition to reflect the food consumed when a shift in diet occurs (Hobson et al. 1996). Turnover rates may largely differ between tissues and, thus, the time window about which they inform is highly variable: liver and plasma integrate information from days to weeks, muscle and blood cells from weeks to months, and bone from months to years (Caut et al. 2011; Ramos and González-Solís, 2012; Giménez et al. 2016; Vighi et al., 2017). 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, and particularly if the growth rate is constant and predictable along the life of the individual, 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 (e.g. Rooker et al., 2008; Cherel et al., 2009; Ramos and González-Solís, 2012; Borrell et al., 2013; Matthews et al., 2016). The length of the preserved record depends on the persistence of the tissue, which in its turn is affected by the progressive erosion of the distal layers as it occurs with nails, hair or baleen plates, or by the abrupt loss of the whole structure as a consequence of molt, as is the case of feathers or hair or fur in some species.

Baleen plates grow incrementally and therefore sequentially archive the stable isotopic seascape of the water mass in which the whale lives or its variation in feeding regimes or other physiological properties during a period that may span several years, depending on the species (Schell and Saupe, 1993; Mitani et al., 2006; Aguilar et al., 2014). Because many whale species or populations stay during part of their life cycle in unknown geographical destinations or during certain periods or regions they remain inaccessible to sampling, baleen plates provide an invaluable insight into these periods that otherwise would remain obscure. Schell et al. (1989) were the first to analyze stable isotopes along the growth axis of a whale baleen plate, obtaining a temporal record of recent movements and diet. Since then, many studies have replicated this approach to gain information on migration and diet shifts in a variety of baleen whales species (e.g. Best and Schell, 1996; Lee et al., 2005; Caraveo-Patiño et al. 2007; Aguilar et al., 2014; Mathews and Ferguson 2015).

Baleen plates hang from the upper jaw in bilateral rows along the rostrum, number up to few hundred on each side 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 (Fudge et al. 2009). It has been supposed that baleen plates grow and erode continuously (Ohsumi et al. 1958; Robins 1959). Records of variations in thickness in different baleen plates proceeding from a single animal suggest that shorter plates present the same pattern than the longer plates' upper part. Thus, shorter baleen plates seem to be exposed to a greater erosion than longer plates do (Ruud 1940), but this hypothesis has only been confirmed in grey whales (Kasuya and Rice 1970; Sumich 2001).

The color of the plates varies between species but, more importantly, in some baleen whales the coloration of the head is asymmetrical and the color of the baleen plates may vary between different segments of the row or between sides of the mouth. 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-slate, while in the right side the lower jaw is white and the rear two-thirds of

the plates are gray-slate but those on the front third are yellowish (Aguilar, 2009). In the sei whale (*Balaenoptera borealis*), most baleen plates are dark gray but those in the front tend to be whitish (Horwood, 2009). In the dwarf minke whale, *Balaenoptera acutorostrata* (Arnold et al., 2005) 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 (Yamada, 2009).

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 (Tershy and Wiley, 1992). In this context it is reasonable to argue that the various baleen plates may have dissimilar structural composition and that the heterogeneity in size according to position in the mouth may be partially or totally due to differences in the baleen plate growth rates. If this were the case, the pattern of tissue layering, and the amplitude of the oscillations of the stable isotope values along the baleen plate, would differ between plates of different size or color, this is, between different mouth regions or mouth sides. Concern about this issue has been expressed in a some previous studies but never addressed through specifically-designed experiments, thus remaining unresolved (Schell et al. 1989, Caraveo-Patiño and Soto 2005; Lubetkin et al. 2008, Bentaleb et al. 2011; Eisenmann et al. 2016).

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 values and their oscillations along the plates. With this objective we have examined the replicability of stable isotope patterns between the baleen plates of a same individual 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 (Aguilar, 2009). Undoubtedly reflecting this, clear oscillations of the stable isotope values in their baleen plates have been observed (Bentaleb et al., 2011; Ryan et al., 2013; Aguilar et al. 2014). In addition, the fin whale is the mysticete in which the coloration of baleen plates shows the highest heterogeneity and asymmetry (Aguilar, 2009), 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 Figure 1 for exact positions). A further 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 in deep freeze. Once at the laboratory, they were thawed, the gum was removed with a 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

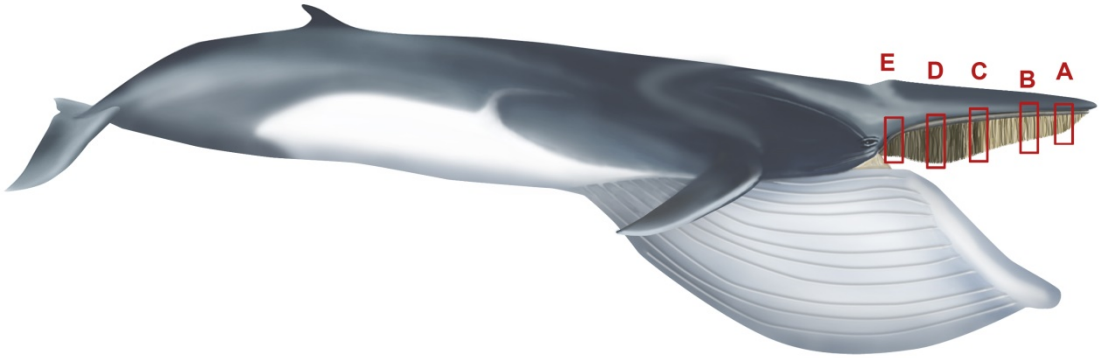

Figure 1: Place of sampling of each plate. The total length of the filtering apparatus was 362cm. The five plates from the right maxilla were collected from roughly equidistant positions from the tip: A at 45cm, B at 90cm, C at 180cm, D at 270cm, and E at 316cm. Plate O was collected from the left maxilla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

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 an optimum range of reference values. The reference materials used are distributed by the International Atomic Energy Agency (IAEA).

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

$$\delta X = [(R_{sample}/R_{standard}) - 1] \times 1000,$$

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, as certified by the International Atomic Energy Agency (IAEA, Vienna). 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 signals of carbon and nitrogen were individually examined by fitting a generalized additive model (GAM) to the data points using mgcv package (Wood, 2011) in R (R Development Core Team, 2010). For each baleen plate and element, homoscedasticity and normality of the residuals were checked, and models were adjusted by removing outliers and choosing best k.

Differences between the mean values of $\delta^{15}N$ and $\delta^{13}C$ of the different baleen plates 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 (Aguilar et al., 2014). Data were tested for normality (Shapiro-Wilk test) and homoscedasticity (Levene's test). For each element, a Kruskal-Wallis test was performed.

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. All baleen plates showed oscillations in their C and N isotope values along their growing axis (Figure 2). 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 $\delta^{15}N$ ($p = 0.57$), when comparing the first 18 cm of each baleen plate.

Table 1: Characteristics of the plates analyzed in this study and δ15N and δ13C ratios (mean ± s.d., permil) for the first 18 cm.

Plate	Pigmentation	Samples	$\delta^{15}N \pm$ s.d.	$\delta^{13}C \pm$ s.d.
A	Yellowish	20	9.7 ± 0.7	-18.5 ± 0.5
B	Yellowish	29	9.6 ± 0.6	-18.8 ± 0.5
C	Grey	45	9.4 ± 0.7	-19.0 ± 0.4
D	Grey	34	9.5 ± 0.6	-19.0 ± 0.4
E	Slate Grey	34	9.8 ± 0.6	-18.9 ± 0.4
O	Grey	44	9.5 ± 0.7	-18.9 ± 0.4

Figure 2: GAM models fitted to the fluctuations in $\delta^{15}N$ and $\delta^{13}C$ values of the baleen plates A - E (a, c panels) and C and O (b, d panels).

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 animals hair, pinniped

vibrissae or bird feathers (e.g. Schwert et al. 2003; Ramos and González-Solís, 2012; Grecian et al. 2015; Cardona et al., 2017). 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 studies of this nature despite expressed concerns about the potential non-replicability between baleen plates from the same individual. Schell et al. (1989) and Lubetkin et al. (2008) investigated oscillations between two opposite plates in a bowhead whale (*Balaena mysticetus*), and Caraveo-Patiño and Soto (2005) compared two consecutive plates in a grey whale (*Eschrichtius robustus*). In both 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. (2016) in humpback whales (*Megaptera novaeangliae*) and that of Bentaleb et al. (2011) 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.

In the present study, the stable isotope values observed the baleen plates of different sizes and sampled in different positions along the filtering apparatus presented nearly identical oscillations (Figure 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 values demonstrate 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.

Another potential source of heterogeneity in sampling between plates is coloration. As mentioned, a number of mysticetes show some degree of asymmetry in body pigmentation, and different segments of the filtering apparatus may show dissimilar plate coloring. The potential effect of skin pigmentation on the stable isotope values has been investigated in a number 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 dolphins (*Stenella coeruleoalba*) (Arregui et al. 2017), bottlenose dolphins (*Tursiops truncatus*) and killer whales (*Orcinus orca*) (Williams et al. 2008), 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 $\delta^{13}C$ ratios (Minagawa 1992; O'Connell and Hedges 1999), all indicating that coloration *per se* should not be expected to have any effect on stable isotope values.

The asymmetrical coloration of both the rostral region and the baleen plates occurring 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 (Mitchell, 1972; Caro et al., 2011), although this hypothesis does not appear to be clearly supported by field data (Tershy and Wiley, 1992). If the asymmetrical variation is limited to pigmentation, the above evidences from skin and hair would point to a non-effect on stable isotope values 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 dissimilar 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 values and their oscillation patterns were satistically indistinguishable between plates C and O, this is, between plates sampled in the same position of the filtering row but collected from opposite sides of the mouth and therefore displaying contrasting coloration.

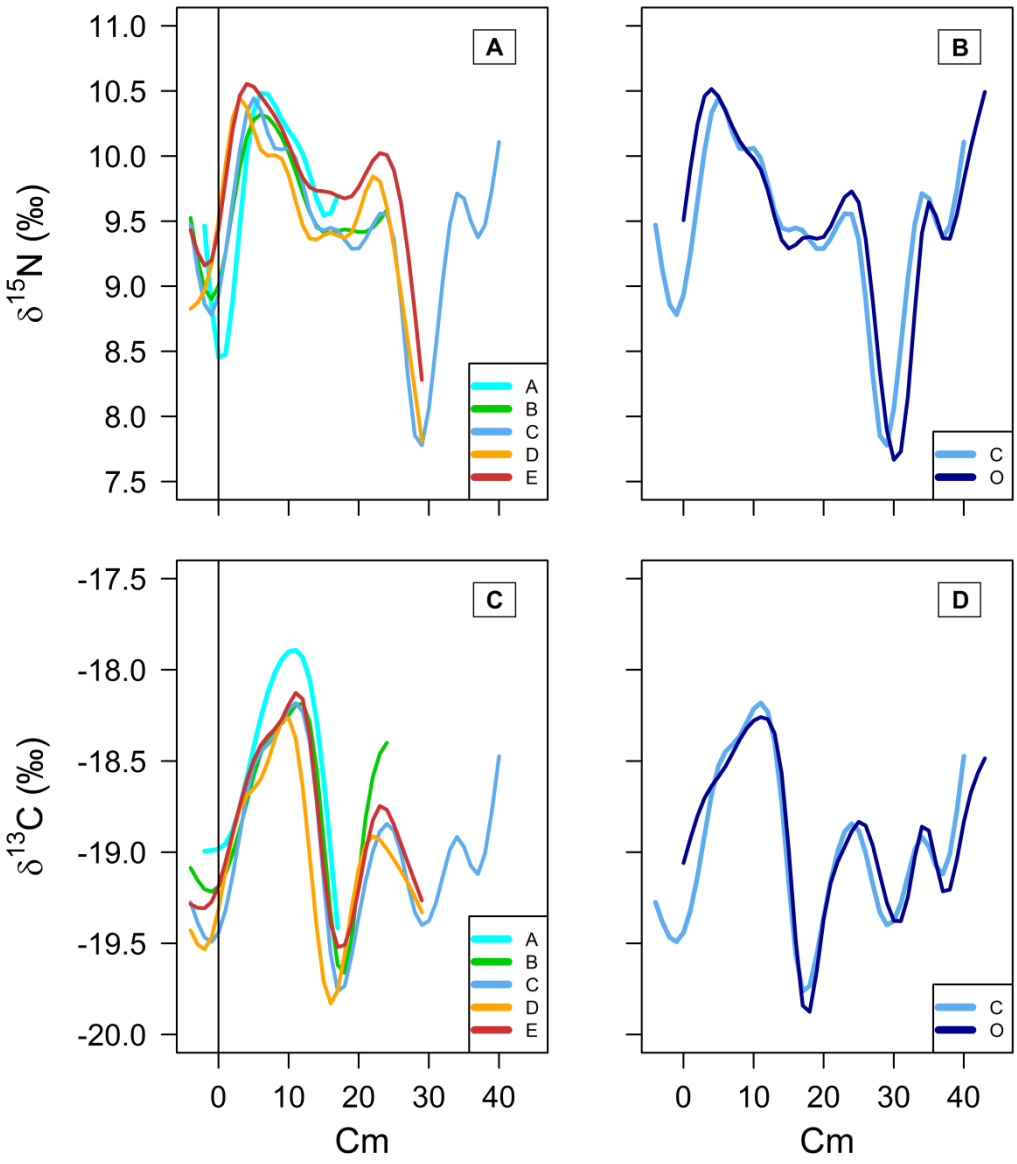

Figure 2.- Oscillations of δ15N (A and B panels) and δ13C values (C and D panels) along the growing axis of the various plates from the left row (A and C panels), and comparison of plates occupying central positions in each body side: C in the left side and O in the right (B and D panels).

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 values 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 stable isotope studies. However, 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 thus representative of the largest number of baleen plates in a filtering apparatus, and are among the largest in the filtering apparatus, thus providing the longest time span for investigating seasonal oscillations.

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