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INTERNATIONAL WHALING COMMISSION

Stable isotope analysis of fecal material provides insight into the diet of baleen whales

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ABSTRACT
In cetaceans, determination of diet through the examination of stomach content or feces is subject to difficulties and potential biases. Application of stable isotope analysis of tissues to diet assessment overcomes some of these caveats but, because tissue isotopic turnover is slow, resultant values are only indicative of long-term feeding and may be misleading if recent shifts in prey consumption have occurred. Determination of stable isotope values in feces stand as a potential alternative source for resolving short-term diet. However, values may be potentially biased by the action of digestive enzymes and bacteria. Here we investigate whether the fecal stable isotope values of freshly dead fin whales are consistent with those of the main food found in their stomachs (krill) as well as with those of other potentially consumed prey from the same ecosystem. Results show that stable isotope values of krill remain unaltered despite their transit along the digestive tract and, therefore, values in feces are reliable indicators of the consumption of this prey. Also, the stable isotope values of feces which under visual inspection appeared to only contain fish remains revealed that contribution of krill in the digested food was substantial, as the δ13C and δ15N values were too low to justify an exclusively piscivorous diet. This demonstrates that results from macroscopic gross analysis of feces may be misleading because less digestible components, such as krill, may be overrepresented. We conclude that stable isotope values of feces contribute significant information to other techniques for assessment of short-term diet reconstruction.

INTRODUCTION
Diet has been traditionally studied through stomach content and gross fecal analyses. These techniques allow precise determination of the prey species consumed but are subject to a number of limitations: i) they inform about diet on a short timescale because the gut contents only hold the last feeding bouts; ii) the diet composition inferred from them may be biased because of differences in prey retention, digestion and degradation rates; and iii) for many species these techniques may only be carried out on dead animals and, if these have dead by natural causes, the results may not be representative of the healthy, standard population (Pierce and Boyle 1991; Kelly 2000; Tollit *et al.* 2010; Bowen and Iverson 2013).

More recently, reconstruction of diet has been conducted through the analysis of stable isotopes in the tissues of the consumer and its potential prey. The technique is based on the principle that the stable isotope composition of the consumer tissues reflects that of the source after some metabolic processing that can be predictably modelled. Most of the studies are based on the stable isotopes of carbon and nitrogen (measured as $\delta^{13}C$ and $\delta^{15}N$ values, respectively), not only because these values are informative of diet but also because their ecological variation and physiological processing are well known (DeNiro and Epstein, 1978, 1981). Stable isotope analysis can be conducted in peripheral tissues, such as skin, nails or hair, that can be non-destructively sampled from live individuals (Kelly 2000; Tollit *et al.* 2010). However, to obtain accurate diet estimations from tissues, interpretation of stable isotope values requires estimation of the food-consumer fractionation (Δ), which is the difference between the isotopic signal of the food and that of the consumer's tissue caused by the absorption and physiological processing of the food materials in the consumer's body (Caut *et al.* 2009). Fractionation is typically calculated through controlled-feeding experiments, but such experiments may not be possible or

extremely difficult in species, such as most cetaceans, that cannot be held in captivity (Salvarina *et al.* 2013).

In the last decades, feces have also been used as an alternative material to investigate short-term diet through stable isotope analysis in wild species (Salvarina *et al.* 2013). Feces can be obtained easily and non-invasively because they do not require killing, capturing or stressing the individual (Sponheimer *et al.* 2003; Codron *et al.* 2012; Salvarina *et al.* 2013). Stable isotope analysis of feces may offer greater accuracy and more reduced laboratory effort than macroscopic fecal analysis (Hatch *et al.* 2011) and, because the ingested material is not physiologically processed, it does not require estimation of prey-consumer fractionation (Δ). However, the stable isotope values in feces may be biased by two factors: the over-representation of body components of certain prey that are resistant to digestion or intestinal absorption, and potential alteration of the fecal isotopic values as a consequence of bacterial activity (Codron *et al.* 2012; Hatch *et al.* 2011).

Although some researchers consider that the significance of these factors is limited and that feces represent better than tissues the isotopic values of the ingested food (Salvarina *et al.* 2013), it is also true that the results of diet-controlled studies have produced disparity of results (e.g. Codron *et al.* 2011, 2005, 2012; Norman *et al.* 2009; Hwang *et al.* 2007; Salvarina *et al.* 2013). This, enhanced by the difficulty in conducting controlled experiments in many wild species, represents a limitation towards the widespread application of fecal stable isotope analysis to determine diet.

Cetaceans are in most cases elusive and difficult or impossible to handle. Moreover, in most species, fresh corpses in which to examine stomach contents are not available unless they originate from stranded individuals that may not be representative of the standard population. Consequently, analysis of stable isotopes values in tissues has been extensively used to achieve this end (Newsome *et al.* 2010) and the food-consumer fractionation for the different tissues assessed (Borrell *et al.* 2013). However, tissue isotopic turnover is slow and, when there is a shift in prey consumed, the steady-state equilibrium between the stable isotope values of the tissue and the diet may take at least several weeks to be reached (Giménez *et al.* 2016).

For these reasons, analysis of stable isotopes of feces stand as a potential source for resolving diet composition of these animals, particularly when recent shifts in prey consumption are suspected or known. Because many species defecate at the surface prior to diving, fecal samples can be collected during the conduction of photo-identification, behavioral or population estimation surveys (for a review, see Hunt *et al.* 2013). Also, scent-detection dogs have been used with good results to improve the location of floating feces (Rolland *et al.* 2006; Ayres *et al.* 2012). The consistency of fecal samples varies with species and diet, invoking a potential sampling bias at sea, but they have proved suitable for conducting stable isotope analyses (Gendron *et al.* 2001; Marcoux *et al.* 2007). However, despite the potential of the technique, no studies have so far been carried out on the potential biases in stable isotopic values introduced by the effects of the enzyme and bacterial activity in the digestive tract because of the impossibility of conducting feeding-controlled experiments. The aim of the present study is to deepen into this subject and examine in freshly dead fin whales (*Balaenoptera physalus*) whether the fecal isotopic values are consistent with those of the main food found in the stomach of the whales, as well as with those of other potentially consumed prey in the ecosystem. This is a necessary step towards validating the stable isotope analyses of feces as a technique to infer short-term diet in these animals.

MATERIAL AND METHODS

Determinations of stable isotope values were conducted on different sets of samples of fecal material from fin whales from Iceland as well as from their known or potential food: i) fecal material obtained from the distal segment of the large intestine of four fin whales; ii) whole body homogenates of prey extracted from the forestomach (first compartment) of 17 fin whales (*Balaenoptera physalus*); iii) whole body homogenates of two euphausiid species (*Meganyctiphanes norvegica* and *Thysanoesa inermis)* known to be consumed by fin whales; and, iv) musche tissue from five fish species (*Mallotus villosus*,

Clupea harengus, *Pollachius virens*, *Melanogrammus aeglefinus* and *Gadus morhua*) which are opportunistic or potential prey of fin whales in these waters.

The whales sampled for krill and feces had been caught by commercial whaling operations and flensed at the factory Hvalur H/F, located in Hvalfjordur, western Iceland (Table 1). The samples were collected during the whaling seasons of 2009 (forestomach krill samples from three whales), 2010 (forestomach krill samples from three whales), 2013 (forestomach krill samples from seven whales) and 2015 (forestomach krill samples and fecal material from the end part of the large intestine from four whales). From one of the latter individuals (F15087), two samples of fecal material were collected from different locations of the large intestine, one extracted more cranially and the other more caudally. All samples were stored at -20°C until analysis.

At the laboratory, the fecal samples were studied macroscopically to assess color and presence of solid elements, and examined under the binocular microscope to identify food remains. Later, forestomach krill and fecal samples were placed in Petri´s dishes and dried at 40°C for 24h. After drying out, they were powdered with mortar and pestle. As lipids are depleted in ^{13}C and, therefore, the overall $\delta^{13}C$ value of a tissue is affected by lipid content, lipids in the sample powder were extracted with a chloroform/methanol (2:1) solution through repeated treatments until the lipophilic fraction was eliminated. After completing the treatment, the samples were again dried out and a subsample of approximately 1mg was weighed into a tin capsule and combusted at 900°C. Five replicas were analyzed from each of the forestomach and fecal samples. Isotopic analyses were carried out by means of analyzer/isotope ratio mass spectrometry (EA-IRMS) using a 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. These analyses were conducted in the Centres Cientifics i Tecnològics of the University of Barcelona (CCiT-UB).

The data from whole body homogenates of the potential prey were obtained from a previous study on the feeding ecology of the minke whale (*Balaenoptera acutorostrata*) and published by Ólafsdóttir *et al.* (2013). Interested readers are referred to this publication for details on sampling and analytical methods.

The variation in the stable isotope values of the fecal material, the forestomach krill and the potential fish prey was examined through the calculation of isotopic standard ellipse areas using the SIBER package (Stable Isotope Bayesian Ellipses in R, Jackson *et al.* 2011) contained in the 3.3.0 version of R.

RESULTS AND DISCUSSION

In this study, the forestomach content of the fin whales examined was krill in all cases, a result consistent with previous surveys that showed that the diet of fin whales in Icelandic waters is almost only composed of the euphausiid *Meganyctiphanes norvegica* (Sigurjónsson and Víkingsson, 1997;

Víkingsson 1997). For the stable isotope analyses we sampled the krill directly from the forestomach of the examined whales because in this way we ensured that the euphausiids belonged to the cohort, and originated from the foraging grounds, on which the fin whales rely. In this way we were confident that these krill reliably reflected that consumed by the whales.

The potential effect of digestion or autolysis on stable isotope values of the feces was a first source of concern. The forestomach of baleen whales consists of non-cornified and non-glandular tissue and, despite the fact that the presence of volatile fatty acids and significant levels of bacteria indicates that some pre-digestive fermentation occurs in it, it is generally believed that actual digestion starts in the following chamber, the fundic stomach (Herwig *et al.* 1984; Herwig and Staley, 1987). In the present study, the whales were all examined within 24 hours post-mortem and their stomach content was only sampled when it had fresh appearance. For this reason, it is presumed that the krill had not been subject to significant digestion. Indeed, the stable isotope values determined in it were consistent with those determined in live krill collected with sampling nets in previous studies around Iceland (Thompson *et al.* 1999; Pétursdóttir *et al.* 2008, 2012).

As a consequence of the preceding considerations, the stomach contents and the associated stable isotope values here determined are considered to reliably represent the diet of the fin whales and, together with the data on other potential euphausiids and fish prey published by Ólafsdóttir *et al.* (2013), taken as the baseline against which to compare the stable isotope values determined in the fecal material.

Comparisons between ingested prey and feces in other species have resulted in somewhat heterogeneous conclusions. Most experiments on large herbivores fed on a controlled diet show that the diet-feces fractionation of $\delta^{13}C$ and $\delta^{15}N$ is consistently negligible. They also allow to distinguish between different feeding strategies (C3-feeders, C4-feeders and mixed-feeders), and to conclude that stable isotope values in feces accurately reflect recent diet (Codron *et al.* 2011, 2005, 2012; Norman *et al.* 2009; Sponheimer *et al.* 2003; Wittmer *et al.* 2010). Studies in wild carnivores and omnivores such as hyenas (Codron *et al.* 2005), bears (Hatch *et al.* 2011), and bats (Lam *et al.* 2013; Painter *et al.* 2009) could not be conducted under controlled-diet conditions, but inferences from their known overall diet led to similar conclusions. However, some studies have alerted about generalization of these conclusions to all species. Hwang *et al.* (2007) found that the feces of small herbivores were significantly enriched in ^{15}N (2.5‰) and depleted in ^{13}C (-3.5‰) as compared to values from a controlled diet, and Salvarina *et al.* (2013) found that the $\delta^{13}C$, $\delta^{15}N$ $\delta^{34}S$ values of feces from two species of captive bats were generally unaffected by digestion except for one species, in which $\delta^{15}N$ showed limited, but statistically significant, enrichment. It has been hypothesized that these species-specific variations may be related to specificities in the metabolism and digestive physiology.

Whatever the case, the potential alteration of the stable isotope values along the digestive tract would essentially depend on two linked processes: the break-down action of bacterial and digestive enzymes and the selective absorption of the light stable isotopes versus the heavy stable isotopes. The two processes are likely to be influenced by the type of food and the structure and function of the digestive tract, so it may be argued that some differences may be expected between herbivores, carnivores and omnivores.

As cetaceans evolved from terrestrial artiodactyls, they retain many similarities with that group of animals in their digestive system and processes. Thus, cetaceans have a multi-chambered stomach (Sanders *et al.* 2015) that increases the passage time of food through the gut and allows more time for microbial and enzymatic digestion (Mårtensson *et al.* 1994). However, Pérez *et al.* (2016) found that the volume of the stomach of baleen whales is much smaller than that of herbivorous artiodactyls, and this may suggest a higher digestibility in the first group of animals and a potential enhancement of effects on the stable isotope values of the food by them ingested. Also, studies on minke whales have proven the existence of chitinase-producing bacteria in the forestomach of this species, a fact that is likely to be also true in other species of baleen whales, including the fin whale. These bacteria are thought to perform the microbial fermentation that permits the digestion and subsequent absorption of chitin, the

tough and resilient protein polymer that conforms the krill exoskeleton (Mårtensson *et al.* 1994; Nordøy *et al.* 1993). From a physiological standpoint, this would parallel the digestion of cellulose by herbivores. Moreover, baleen whales have an exclusive capacity among mammals to digest wax esters, another resilient component of the dry matter of krill species (Nordøy 1995). All this warned about the need to investigate fractionation between baleen whale diet and feces.

The SIBER ellipses plot (Figure 1), showed a large variation in both the $\delta^{13}C$ and $\delta^{15}N$ values of krill that undoubtedly reflects the heterogeneous diet of euphausiids, which present ontogenetic and seasonal shifts from exclusively phytoplanktonic to omnivorous-carnivorous feeding (Schmidt 2010). However, despite this large variability, krill stable isotope values were much lower both for C and N than corresponding values from the other potential prey. The values of the fecal material from whales F15085, F15086 and F15088, which in all cases only showed fragments of euphausiid exoskeletons and presented an orange-pink-brown coloration resembling that of krill, fell well within the limits of the *M. norvegica* ellipse. As a consequence, a first conclusion of the study can be that stable isotope values of krill remain unaltered despite their transit along the digestive tract and that fecal values are demonstrative of consumption of this type of prey. However, the two fecal samples of individual F15087, both collected in the large intestine although one more cranially (F15087cr) than the other (F15087cd), showed values that were extralimital to the convex hull of krill (Figure 1). Under the binocular microscope the fecal material showed to contain both fragments of euphausiid exoskeletons as well as abundant skull fragments, spines and vertebrae of fishes (Table 1). This demonstrated fish consumption, a fact previously observed sporadically in fin whales off Iceland (Sigurjónsson and Víkingsson 1997). It should be highlighted that in both cases these feces presented a darker and greenish coloration and the only macroscopic remains that could be identified with the naked eye were fish bones. Only the examination under the binocular microscope revealed some krill remains. However, the fact that the stable isotope values of the fecal material fell right in the middle between the values of krill and those of herring, the fish species displaying closest values to those of krill, indicates that the contribution of krill in the feces was indeed significant, something that otherwise may have been overlooked.

It is unclear, however, whether the stable isotope values of fish may have been altered by the effects of selective absorption, digestion or the bacterial activity that characterizes the internal environment of the digestive tract. This could be only be ascertained through feeding-controlled experiments, but these are difficult or impossible to carry out for most cetacean species.

However, even in the absence of such studies the stable isotope analysis of fecal material from free-ranging whales stands as a useful tool to assess diet. The technique is devoid of the usual drawbacks that hinder stomach content analysis of naturally stranded whales, which are often empty, may be biased towards species of unusual consumption, and are usually subject to severe autolysis (Ryan *et al.* 2013). The macroscopic examination of fecal remains overcomes these caveats and allows in some cases precise identification of species, but may be ignoring prey that have disintegrated during their transit along the digestive tract and thus may overestimate prey with less digestible anatomic components. Microscopic examination improves prey identification, but the prevalence of the species involved may be impossible to determine. Stable isotope values of feces would provide a complementary insight to the macroscopic and microscopic examinations by alerting about prey impossible to identify in the remains and by informing about potential prevalence of different prey, therefore contributing additional information to short-term diet reconstruction.

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Table 1.- Information on the whales and the fecal samples used in the study.

Code of individual	Gender	Body length (m)	Location of collection of fecal sample	Macroscopic remains	Colour of faeces
F15085	Male	16.85	End part of large intestine	None visible	Orange-brown
F15086	Female	19.90	End part of large intestine	None visible	Brown
F15087	Male	18.82	End part of large intestine	Fish bones present	Dark green
			Anterior part of large intestine	Fish bones present	Dark green
F15088	Male	17.06	End part of large intestine	None visible	Pale brown-green

Figure 1.- Standard ellipses corrected for sample size (SEAc) and convex hulls (polygons encompassing all the data points) for the stable isotope values determined in the main potential prey species of fin whales in Icelandic waters. The red triangles (A-E) depict the stable isotope values of the fin whale feces samples collected in the present study (A: F15085; B: F15086; C: F15087 (anterior part of large intestine); D: F15087 (end part of large intestine); E: F15088. Data on krill (*Meganyctiphanes norvegica*) and fin whale feces were obtained for the present study, while data from the other prey species were extracted from Ólafsdóttir et al. (2013).