Stable Isotope Analysis of Humpback and Fin Whales off Virginia Beach, Virginia

Prepared by Danielle M. Waples

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INTRODUCTION

Stable isotope analysis is a powerful technique that can provide insights into animal habitat use and foraging ecology and is becoming increasingly used in marine mammal studies (Newsome et al. 2010). The stable isotopic composition of an animal's tissues reflects the isotopic composition of its assimilated diet (Hobson 1999; Kelly 2000; Newsome et al. 2010), although stable isotope enrichment (an increase in the abundance of the heavier isotope) occurs between an animal and its food due to physiological processes (DeNiro and Epstein 1978; Mendez-Fernandez et al. 2012). In general, the enrichment in ^{15}N between prey and predator is typically 3-4‰, mainly due to the preferential excretion of ^{14}N (DeNiro and Epstein 1981; Minagawa and Wada 1984; Peterson and Fry 1987). The enrichment in ^{13}C is estimated to be around 1‰ per trophic level due to carbon isotopic fractionation during assimilation or respiration (DeNiro and Epstein 1978; Peterson and Fry 1987). Caut et al. (2011) fed a captive killer whale (*Orcinus orca*) a long-term controlled diet and calculated enrichment factors of 0.09‰ for ^{13}C and 3.05‰ for ^{15}N in a skin sample.

The enrichment in ^{15}N between trophic levels is relatively large and predictable and can be used to identify an animal's trophic position (Minagawa and Wada 1984; Fry 1988; Hobson and Welch 1992; Rau et al. 1992; Lesage et al. 2001). The enrichment in ^{13}C along the food chain is smaller and more variable than ^{15}N enrichment but provides useful insight into sources of primary production (Rau et al. 1992; Lesage et al. 2001). Thus, carbon isotopes can provide information about foraging habitat, based on inferences regarding the sources of carbon (Ramsay and Hobson 1991; France 1995; Smith et al. 1996; Clementz and Koch 2001). Cetacean skin has a tissue turnover rate of approximately two to three months (Hicks et al. 1985) and isotopic values reflect diet assimilated during that period.

Isotope ratios are expressed in delta (δ) notation as parts per mil (‰) where δ is the ratio of the sample relative to a standard:

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

in which X is the element, *h* is the atomic mass of the heavy isotope and R_{sample} and $R_{standard}$ are the heavy to light isotope ratios ($^{13}C/^{12}C$ or $^{15}N/^{14}N$) of the sample and standard, respectively (Newsome et al. 2010). The accepted standards are carbonates from Vienna Pee Dee Belemnite limestone for $\delta^{13}C$ and atmospheric nitrogen for $\delta^{15}N$ (Newsome et al. 2010).

Humpback whales (*Megaptera novaeangliae*) in the western North Atlantic are known to migrate between high-latitude summer feeding grounds and low-latitude winter breeding grounds (Clapham et al. 1992). However, some humpback whales have been documented off Virginia during the winter months and it has been suggested that they are juveniles using this area as a

winter feeding ground (Swingle et al. 1993; Barco et al. 2002). The objective of the current project was to conduct stable isotope analyses on biopsy samples collected from humpback whales off Virginia during the winter months to characterize their isotopic signatures.

MATERIAL AND METHODS

Sample Collection

HDR personnel collected samples from humpback and fin (*Balaenoptera physalus*) whales off Virginia from January 2015 through January 2017. Biopsies were collected from either a 68-kg pull Barnett crossbow equipped with 25-mm sterilized stainless steel tips or from a Paxarms biopsy rifle firing 6x20-mm dart tips using .22 caliber blank cartridges (Aschettino et al. 2016). The skin was excised from the blubber, separated into three subsamples and the portion for stable isotope analysis was stored in a cryovial and frozen at -40°C. Samples were transported to Duke University Marine Lab and stored in a -20°C freezer until sample preparation.

Sample Preparation and Stable Isotope Analysis

Skin samples were dried in an oven at 60°C for 48 hours and then homogenized to a fine powder. Compared to proteins and carbohydrates, lipids are depleted in ^{13}C and thus typically have more negative $\delta^{13}C$ values that can bias stable isotope analyses (DeNiro and Epstein 1977, Post et al. 2007, Borrell et al. 2012, Ryan et al. 2012). We lipid extracted all the samples using a chloroform and methanol solvent (2:1 v/v) following the protocol of Folch et al. (1957) and Lesage et al. (2010). Approximately 20-40mg of dried and homogenized samples were placed in glass tubes with 1ml of solvent, agitated for 10 minutes on a Multi-Pulse Vortexer and stored overnight. The solvent was removed the next day via pipette and a fresh 1ml of solvent was added. We repeated this procedure three times and after the final removal of the solvent the samples were stored overnight in a fume hood to dry via evaporation. Lipid extraction can cause unpredictable changes in $\delta^{15}N$ values (Sotiropoulos et al. 2004; Lesage et al. 2010; Ryan et al. 2012), therefore we analyzed a portion of each skin sample for $\delta^{15}N$ prior to lipid extraction and the remainder of each sample was analyzed for $\delta^{13}C$ after lipid extraction was completed. Approximately 0.7-1.2 mg of each dried and homogenized sample was sealed in 8x5mm tin capsules. Stable isotope analyses were performed at the Duke Environmental Isotope Laboratory in Durham, North Carolina via a continuous flow mass spectrometer system (Thermo Finnigan Delta Plus XL). The external precision relative to reference materials was approximately ± 0.1‰ for both $\delta^{13}C$ and $\delta^{15}N$.

We examined the effects of lipid extraction on stable isotope values using a paired t-test. We also examined inter-species differences and potential differences in humpback whale stable isotope signatures caused by gender with Student's t-tests using JMP 13.0 statistical software.

Genetic Analysis

Whale skin samples were subsampled and finely chopped using a scalpel blade for a final mass of approximately 15mg. DNA was extracted by silica spin column using the Wizard SV Genomic DNA purification system (catalog no. A2360) and stored at -20°C until ready for polymerase chain reaction (PCR) amplification. For gender determination, we performed

multiplex PCR to amplify a 447bp segment on the X chromosome (forward primer: 5'-GCACCTCTTTGGTATCTGAGAAAGT-3', reverse primer: 5'-ACAACCACCTGGAGAGCCACAAGCT-3') and a 224bp segment on the Y chromosome (forward primer: 5'-CCCATGAACGCTTTCATTGTGTGG-3', reverse primer: 5'-CTCTTGGCCTTCCGACGAGGTCGATA-3'). Primers were based on the p2-3ez/p1-3ez (Aasen and Medrano 1990) and Y53-3C/Y53-3D (Fain and LeMay 1995) systems with slight modifications to reflect recent mysticete whale sequences. We modified the p2-3ez/p1-3ez primers to match a *Balaenoptera acutorostrata scammoni* predicted ZFX gene sequence (GenBank accession no. XM_007185147, Yim et al. 2014) and the Y53-3C/Y53-3D primers to match a *Megaptera novaeangliae* SRY gene sequence (GenBank accension no. AB108513.2, Nishida et al. 2007). PCR was carried out using a 20µL reaction with final reagent concentrations of 1x PCR buffer, 2.0 mM MgCl2, 200 µM dNTPs, 0.25 µM each primer forward and reverse, and 0.5 U/µL Taq. Thermocycling consisted of an initial four minute denaturation step at 94°C, followed by 35 cycles of denaturation at 94°C for 15 seconds, annealing at 60°C for 15 seconds, extension at 72°C for 30 seconds, and a final extension period of five minutes at 72°C. Amplification products were separated by electrophoresis on a 2% agarose gel and gender was inferred to be male for samples which showed two distinct bands at approximately 447bp and 224bp and female for animals that showed only one distinct band at approximately 447bp. The genetic analysis was conducted at Duke University Marine Laboratory.

RESULTS

HDR personnel obtained skin samples from 29 humpback whales and two fin whales from January 2015 through January 2017. All humpback samples were collected during near shore surveys in the winter between the months of November and February and the two fin whale samples were collected during offshore surveys in April.

We examined the data for outliers, defined by Borrell (2012) as values differing by more than three standard deviations from the overall mean, and identified one humpback whale, (20150122_DTE_Mn_001) sampled on 22 January 2015, as having an outlier $\delta^{13}C$ value (Figure 1). We ran a replicate of this individual's sample and received similar results to the original isotopic values ($\delta^{13}C$ values of -22.8‰ and -22.6‰). This animal also had the lowest $\delta^{15}N$ value of any of the humpback whales sampled (Figure 1) and a similar value occurred in both the original sample and the replicate sample ($\delta^{15}N$ values of 12.5‰ and 12.6‰). The values for this humpback were excluded from analyses.

Lipid extraction did have an effect on $\delta^{13}C$ values; the lipid extracted samples were enriched in ^{13}C compared to the non-lipid extracted samples (Figure 2). The differences in $\delta^{13}C$ values between lipid extracted versus non-lipid extracted were significant for both humpback whales ($p<0.001$) and fin whales ($p= 0.004$). However, lipid extraction did not have an effect on $\delta^{15}N$ values (Figure 3). There was no significant difference in $\delta^{15}N$ values between lipid-extracted versus non-lipid-extracted samples for either humpback whales ($p= 0.562$) or fin whales ($p= 0.636$).

The humpback whale skin had a mean $\delta^{13}C$ value of -18.9 (± 0.5) and a mean $\delta^{15}N$ value of 14.6 (± 0.9) (Figure 4). The two fin whales that were sampled had a mean $\delta^{13}C$ value of

-18.1 (± 0.2) and a mean $\delta^{15}N$ value of 10.5 (± 0.0) (Figures 4 and 5). There was a significant difference in $\delta^{13}C$ values between the humpback whale and fin whales ($p= 0.032$) and the humpback whales had significantly higher $\delta^{15}N$ signatures than the fin whales ($p< 0.001$).

Our genetic analyses identified 14 female and 15 male humpback whales sampled during the study period (Table 1). In addition, both of the fin whales that were biopsied were genetically identified as males (Table 1). Females had a mean $\delta^{13}C$ value of -19.1 (±0.4) and a mean $\delta^{15}N$ value of 14.3 (±0.9) while male humpbacks mean values of $\delta^{13}C$ and $\delta^{15}N$ were -18.8 (±0.5) and 14.9 (±0.8) respectively (Figures 6 and 7). There was no significant difference in the $\delta^{13}C$ signature between female and male humpback whales ($p= 0.066$). The females generally had lower $\delta^{15}N$ values than males and this difference was statistically significant ($p= 0.029$).

DISCUSSION

Our results provide the first stable isotope values for humpback whales off Virginia Beach during the winter months. It has been hypothesized that these animals are juveniles that are using the area as a winter feeding ground instead of migrating to lower latitudes (Swingle et al. 1993; Barco et al. 2002). This is corroborated by field observations from Aschettino and colleagues (2016) who categorized 65-76% of the humpbacks they observed off Virginia Beach during the winter as juveniles, based on field size estimates during two years of survey effort.

Our results are comparable to other studies describing stable isotope signatures for humpback whales in other regions (Table 2). Interestingly, our findings are very similar to those of Gavrilchuk et al. (2014) who performed stable isotope analyses on skin samples collected from four rorqual species during the summer months in the Gulf of St. Lawrence when the animals are presumably feeding. They found a mean $\delta^{15}N$ value for humpback whales of 14.3 (± 0.6), compared to our mean $\delta^{15}N$ value of 14.6 (± 0.9). While not conclusive, this does indicate that the humpbacks in both areas are at a similar trophic level and adds support to the idea that humpback whales off Virginia are engaged in feeding during the winter.

We also conducted stable isotope analysis on two skin samples from fin whales that were located and biopsied during offshore vessel surveys in April. The isotopic signatures of these samples are also comparable to those reported for fin whales in other locations (Table 2). They were most similar to Borrell et al. (2012) who sampled fin whales collected during whaling off the northwestern coast of Spain.

There was a significant difference in both $\delta^{13}C$ and $\delta^{15}N$ values between the humpback and fin whales in our study area. The humpback whales were slightly more depleted in $\delta^{13}C$ and had significantly higher $\delta^{15}N$ signatures than the fin whales. The humpback whales had a mean $\delta^{15}N$ value of 14.6 (± 0.9) compared to the fin whales value of 10.5 (± 0.0). Given a difference in $\delta^{15}N$ values between the two species of 4.1‰ it is likely that the humpback whales are feeding at a

higher trophic level than the fin whales in our area. Gavrilchuk et al. (2014) also found that humpback whales occupied a higher isotopic niche than fin whales in their study area.

Our genetic analyses identified 14 female and 15 male humpback whales sampled in the study area. We found no significant differences in δ^{13} values between male and female humpback whales but females did have significantly lower $\delta^{15}N$ values than males. These results are in contrast to findings from other studies (Todd et al. 1997; Gavrilchuk et al. 2014; Fleming et al. 2016) who found no differences between male and female humpback δ^{13} or $\delta^{15}N$ signatures. It should be noted that differences in mean $\delta^{15}N$ values between female and male humpbacks in our study do not reflect a difference in trophic level between males and females as there is less than 1‰ between the mean $\delta^{15}N$ values and a difference in trophic level is typically indicated by a difference of 3-4‰ (Caut et al. 2011). These findings do suggest that the diets of the two sexes may differ in this area.

In conclusion, this project has established baseline stable isotope signatures for humpback and fin whales during the winter months off Virginia Beach and noted inter- and intra-specific differences in those signatures. Future survey and biopsy efforts can be used to supplement these baseline isotopic values and allow for further comparisons to be made.

ACKNOWLEDGMENTS

Will Cioffi conducted the genetic analyses to determine gender and wrote the detailed protocol included in this report. Thanks to David Honig and Philip Turner who provided advice and materials to assist with homogenizing the skin samples. Thanks also to Jennifer Dunn who provided administrative support.

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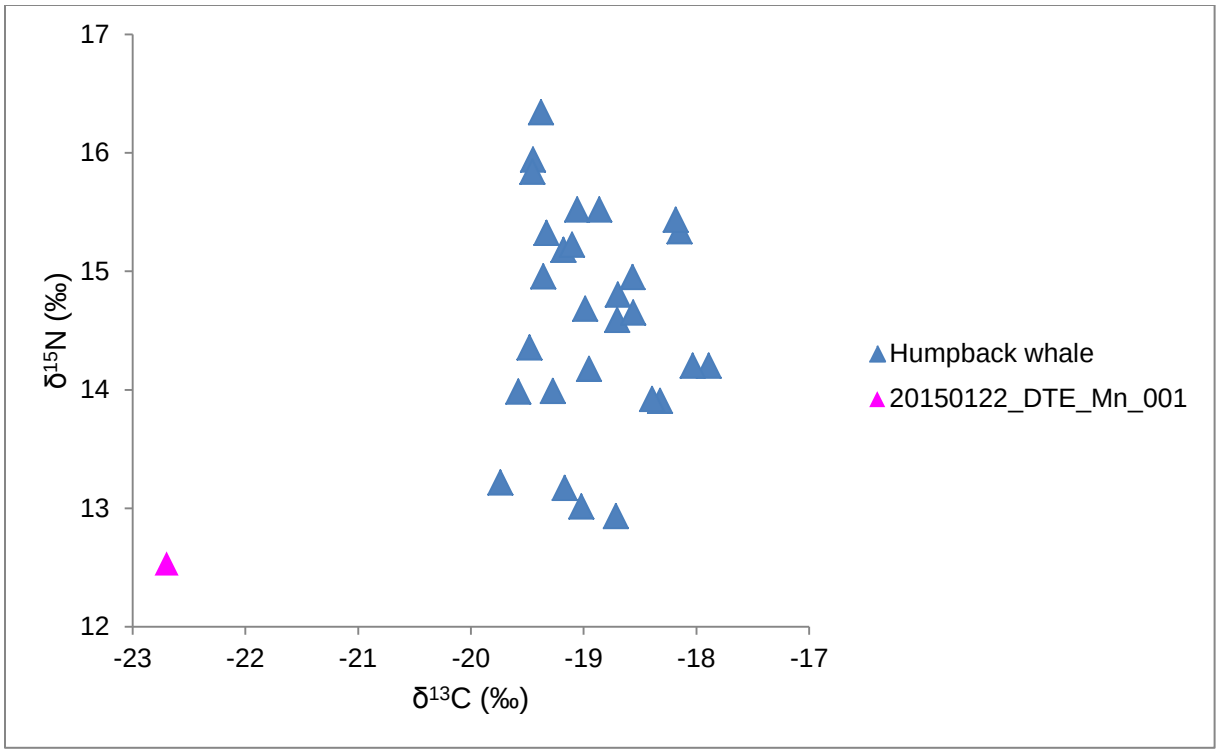

Figure 1. Stable isotope ($\delta^{13}C$ and $\delta^{15}N$) values for humpback whales (n=29) including the outlier values for humpback 20150122_DTE_Mn_001.

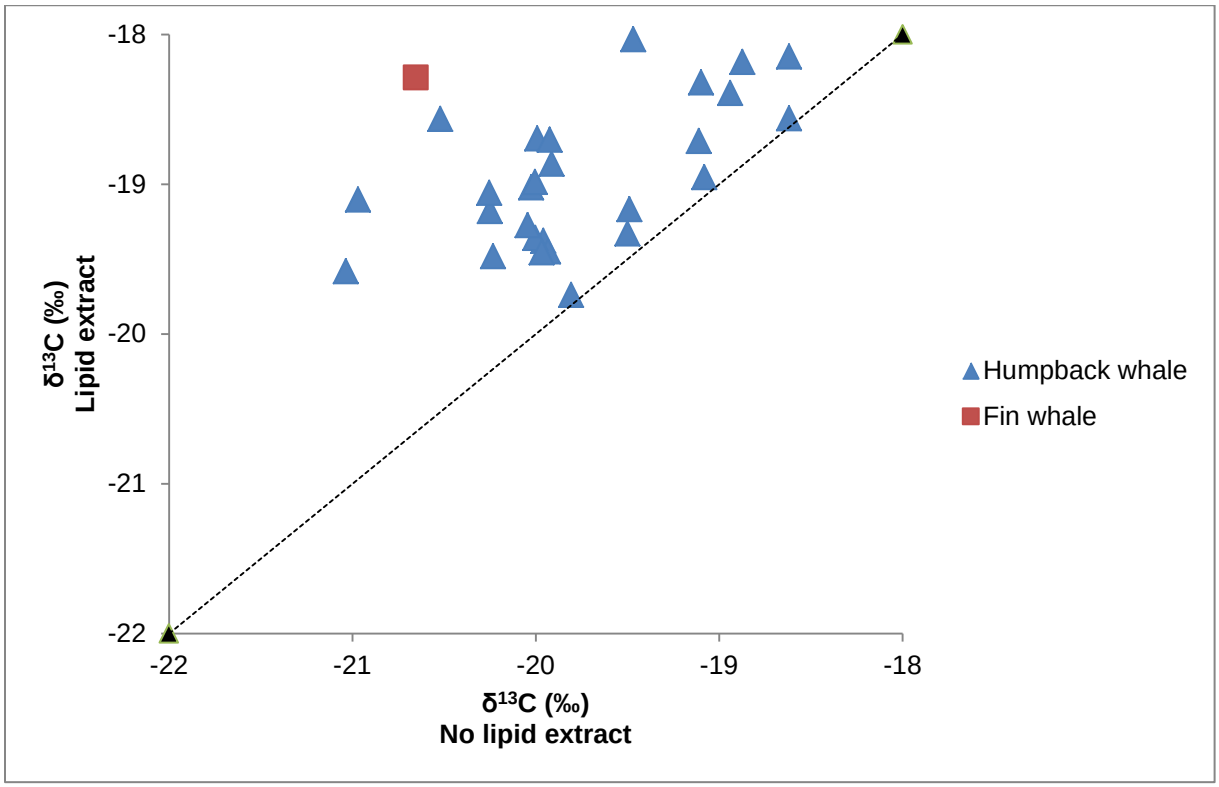

Figure 2. Comparison of non-lipid and lipid-extracted values for $\delta^{13}C$ values for humpback (n=28) and fin (n=2) whales. The dotted line indicates a 1:1 line.

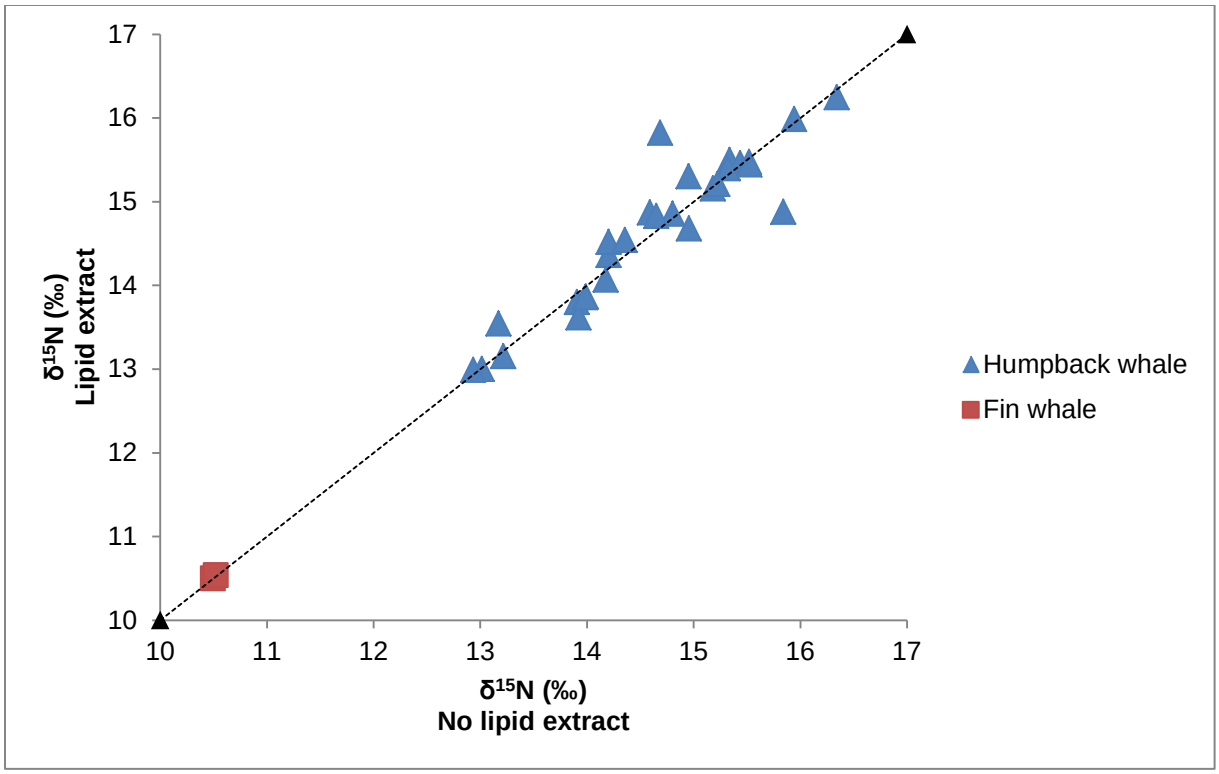

Figure 3. Comparison of non-lipid and lipid-extracted values for $\delta^{15}N$ values for humpback (n=28) and fin (n=2) whales. The dotted line indicates a 1:1 line.

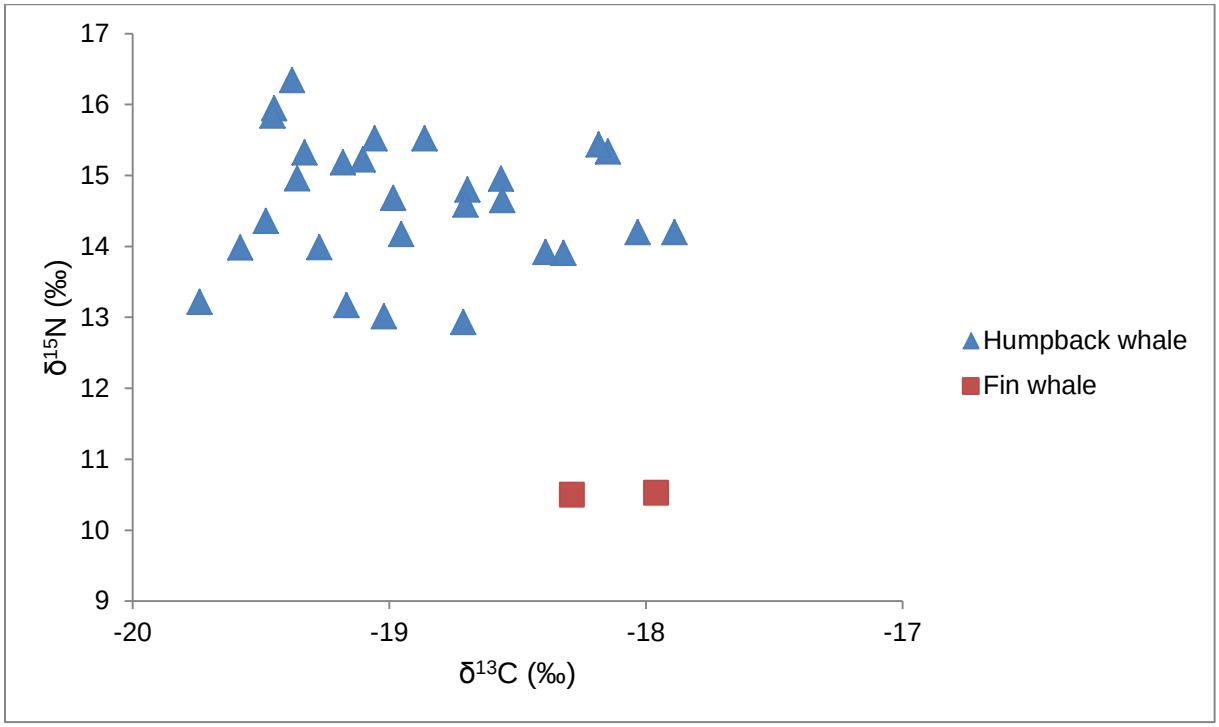

Figure 4. Stable isotope ($\delta^{13}C$ and $\delta^{15}N$) values for humpback whales (n=28) and fin whales (n=2).

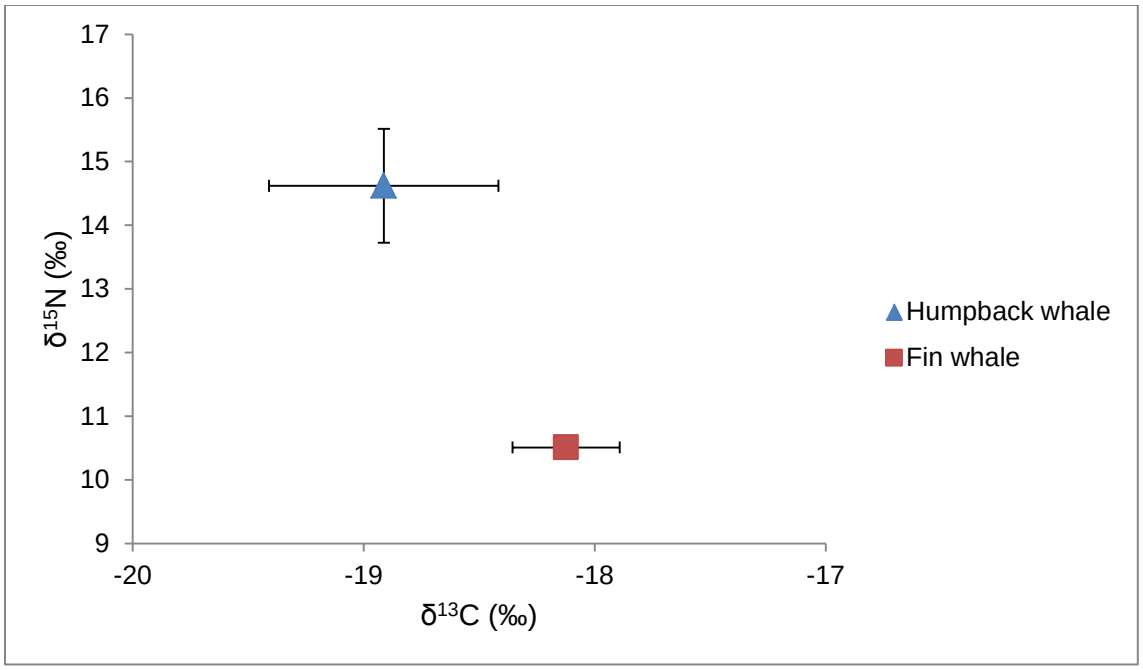

Figure 5. Stable isotope ($\delta^{13}C$ and $\delta^{15}N$) mean (± SD) values for humpback whales (n=28) and fin whales (n=2).

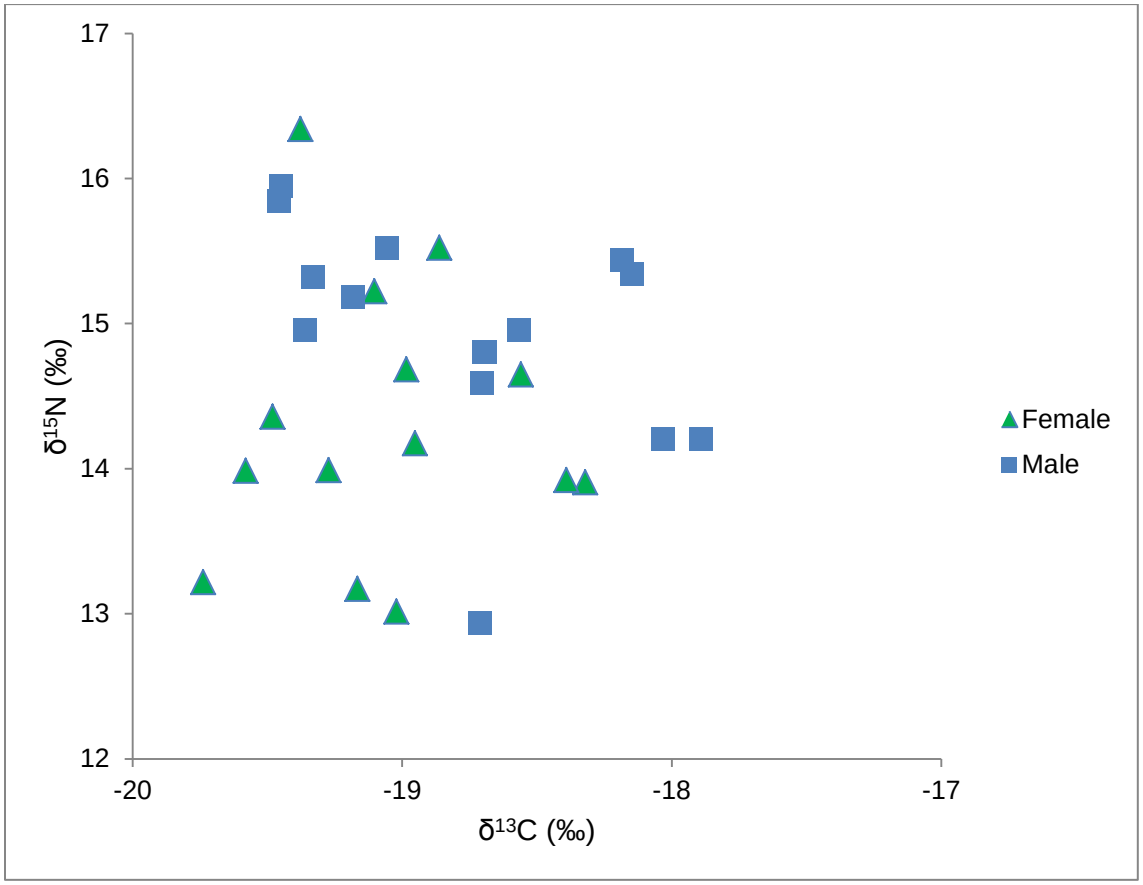

Figure 6. Stable isotope ($\delta^{13}C$ and $\delta^{15}N$) values for female (n= 14) and male (n=14) humpback whales.

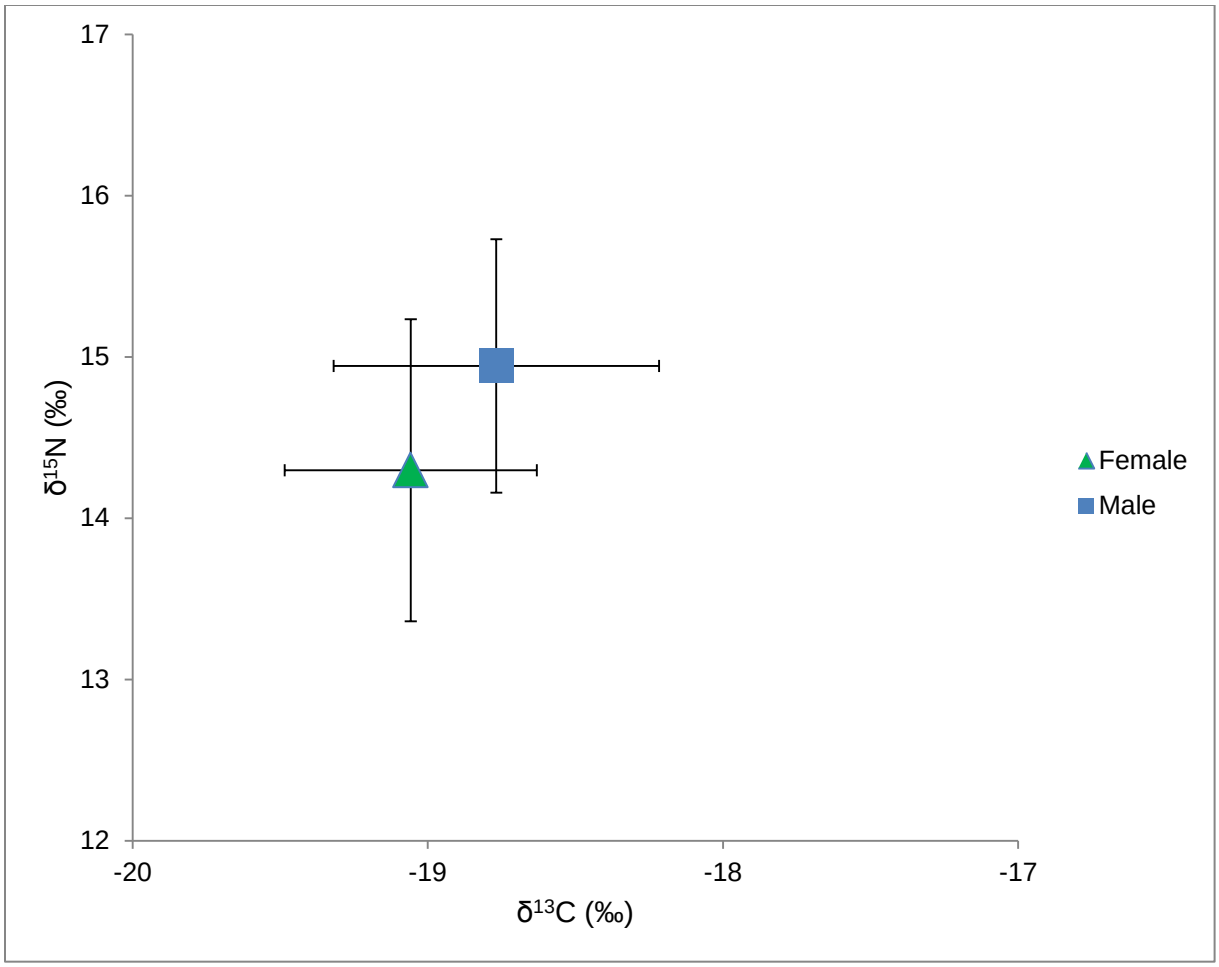

Figure 7. Stable isotope ($\delta^{13}C$ and $\delta^{15}N$) mean (± SD) values for female (n=14) and male (n=14) humpback whales.

Table 1. List of biopsy samples collected by HDR personnel along with sample identification name, species and gender.

Sample Date	Sample ID	Sample Name	Species	Common Name	Gender
02-Jan-15	1	20150102_DTE_Mn_001	*M. novaeangliae*	Humpback whale	Female
06-Jan-15	3	20150106_DTE_Mn_002	*M. novaeangliae*	Humpback whale	Female
11-Jan-15	4	20150111_DTE_Mn_001	*M. novaeangliae*	Humpback whale	Female
11-Jan-15	6	20150111_DTE_Mn_003	*M. novaeangliae*	Humpback whale	Female
22-Jan-15	8	20150122_DTE_Mn_001	*M. novaeangliae*	Humpback whale	Male
22-Jan-15	9	20150122_DTE_Mn_002	*M. novaeangliae*	Humpback whale	Male
29-Jan-15	10	20150129_DTE_Mn_001	*M. novaeangliae*	Humpback whale	Female
29-Jan-15	11	20150129_DTE_Mn_002	*M. novaeangliae*	Humpback whale	Female
2-Feb-15	12	20150209_DTE_Mn_001	*M. novaeangliae*	Humpback whale	Male
29-Apr-15	13	20150429_DTE_Bp_001	*B. physalus*	Fin whale	Male
28-Apr-15	14	20150429_DTE_Bp_002	*B. physalus*	Fin whale	Male
07-Dec-15	15	20151207_DTE_Mn_001	*M. novaeangliae*	Humpback whale	Male
09-Dec-15	16	20151209_DTE_Mn_001	*M. novaeangliae*	Humpback whale	Male
10-Dec-15	17	20151210_DTE_Mn_001	*M. novaeangliae*	Humpback whale	Female
20-Dec-15	18	20151220_DTE_Mn_001	*M. novaeangliae*	Humpback whale	Male
20-Dec-15	19	20151220_DTE_Mn_002	*M. novaeangliae*	Humpback whale	Male
15-Jan-16	20	20160115_DTE_Mn_001	*M. novaeangliae*	Humpback whale	Female
15-Jan-16	21	20160115_DTE_Mn_002	*M. novaeangliae*	Humpback whale	Male
15-Jan-16	22	20160115_DTE_Mn_003	*M. novaeangliae*	Humpback whale	Male
15-Jan-16	23	20160115_DTE_Mn_004	*M. novaeangliae*	Humpback whale	Male
09-Feb-16	24	20160209_DTE_Mn_001	*M. novaeangliae*	Humpback whale	Male
17-Feb-16	25	20160217_DTE_Mn_001	*M. novaeangliae*	Humpback whale	Male
01-Nov-16	26	20161101_DTE_Mn_001	*M. novaeangliae*	Humpback whale	Female
01-Nov-16	27	20161101_DTE_Mn_002	*M. novaeangliae*	Humpback whale	Male
03-Nov-16	28	20161103_DTE_Mn_001	*M. novaeangliae*	Humpback whale	Male
03-Nov-16	29	20161103_DTE_Mn_002	*M. novaeangliae*	Humpback whale	Male
18-Nov-16	30	20161118_DTE_Mn_001	*M. novaeangliae*	Humpback whale	Female
13-Dec-16	31	20161213_DTE_Mn_001	*M. novaeangliae*	Humpback whale	Female
21-Dec-16	32	20161221_DTE_Mn_001	*M. novaeangliae*	Humpback whale	Female
21-Dec-16	33	20161221_DTE_Mn_002	*M. novaeangliae*	Humpback whale	Female
01-Jan-17	34	20170101_DTE_Mn_001	*M. novaeangliae*	Humpback whale	Female

Table 2. Studies reporting stable isotope ($\delta^{13}C$ and $\delta^{15}N$) mean (± SD) values for humpback and fin whales in different locations. All studies reported values based on collected skin samples.

Study	Species	Location	$\delta^{13}C$ (‰)	$\delta^{15}N$ (‰)
Ostrom et al. 1993*	Humpback whale	Newfoundland	-18.7	13.4
Todd et al. 1997	Humpback whale	Western North Atlantic	-18.8 ± 0.1	14.2 ± 0.1
Ryan et al. 2012	Humpback whale	Ireland	-19.6 ± 1.2	12.8 ± 1.1
Gavrilchuk et al. 2014	Humpback whale	Gulf of Saint Lawrence	-18.7 ± 0.4	14.3 ± 0.6
Current study 2017	Humpback whale	Virginia	-18.9 ± 0.5	14.6 ± 0.9
Borrell et al. 2012	Fin whale	Spain	-18.3 ± 0.4	10.0 ± 0.3
Ryan et al. 2012	Fin whale	Ireland	-18.2 ± 0.5	12.0 ± 1.2
Gavrilchuk et al. 2014	Fin whale	Gulf of Saint Lawrence	-18.6 ± 0.4	12.4 ± 1.3
Current study 2017	Fin whale	Virginia	-18.1 ± 0.2	10.5 ± 0.0

* Ostrom et al. 1993 did not report a standard deviation as they only sampled one whale