UNIVERSITY OF CALIFORNIA SAN DIEGO

# Effects of decalcification, species, and mammalian order on bulk stable isotope values from marine mammal teeth

A thesis submitted in partial satisfaction of the requirements for the degree Master of Science

in

Marine Biology

by

Brenna Claire Groom

Committee in Charge:

Professor Carolyn Kurle, Chair
Professor Ron Burton
Professor Brice Semmens

2018

The Thesis of Brenna Claire Groom is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Chair

University of California San Diego

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ABSTRACT OF THE THESIS

Effects of decalcification, species, and mammalian order on bulk stable isotope values from marine mammal teeth

by

Brenna Claire Groom

Master of Science in Marine Biology

University of California San Diego, 2018

Professor Carolyn Kurle, Chair

The stable carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) isotope analysis of marine mammal teeth are useful for reconstructing their past foraging ecology and habitat use, among other things. Fossilized teeth require removal of inorganic compounds via a decalcification process before the stable isotope analysis of the organic collagen. To test for the necessity of decalcification for the proper analysis of the $\delta^{13}C$ and $\delta^{15}N$ values from modern marine mammal teeth, I compared the $\delta^{13}C$ and $\delta^{15}N$ values from decalcified vs. intact dentin sampled from multiple individuals (n=23 total) of seven species of marine mammals. I found no differences in the $\delta^{13}C$ (mean±SD: -

14.1±1.3‰ vs. -14.2±1.2‰) or $\delta^{15}$N (mean±SD: 16.6±2.4‰ vs. 16.7±2.3‰) values from intact vs. decalcified samples, respectively. The C:N ratios were slightly higher for intact (3.0±0.2) vs. decalcified (2.8±0.1) teeth. My results follow those of a previous study examining effects of decalcification from one sperm whale tooth and underscore the recommendation that decalcification is not necessary before the stable isotope analysis of dentin from modern carnivorous marine mammal teeth.

## Introduction

The analysis of stable carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) isotopes has been extremely useful in analyzing historical and modern foraging patterns of marine mammals (Newsome, Clementz & Koch 2010). Ecologists use this technique to determine the life history patterns of animals through reconstructing their diet and migration patterns (Brault et.al). The isotopic analysis of teeth and bones has especially been useful since, once formed, they are inert tissues that grow in layers, or rings, thereby preserving the sequential $\delta^{13}C$ and $\delta^{15}N$ values that reflect an animal's foraging ecology over time (Elorriaga-Verplancken et al. 2013).

The stable isotope analysis of fossilized teeth requires a decalcification process because their organic carbon is potentially contaminated with many different impurities such as biogenic apatite, sedimentary carbonates, and fulvic acids (Clementz, Koch et. al 2001). Therefore, in order to accurately measure the $\delta^{13}C$ and $\delta^{15}N$ values from the organic material in fossilized teeth, separation of the collagen from the nonorganic apatite and other impurities is necessary. Some scientists have questioned whether this process is necessary for modern teeth (Koch et. Al 1997; Brault et al 2014). A recent paper (Brault et al 2014) examined the $\delta^{13}C$ and $\delta^{15}N$ values of intact vs. decalcified samples from growth layers removed from a single sperm whale tooth and found no difference in their isotope values, suggesting this process was unnecessary when preparing modern teeth for stable isotope analysis.

I compared the $\delta^{13}C$ and $\delta^{15}N$ values from intact vs. decalcified teeth from seven marine mammal species to further determine if the decalcification process is necessary for the accurate analysis of stable isotope values from modern marine mammal teeth. If my results support those of Brault et al (2014) in their analysis of one tooth, then the elimination of the decalcification process will save considerable time during the preparation phase of isotope analysis. In addition,

there is frequently very little material within the growth layers of teeth and the loss of dentin with decalcification is high, thereby further reducing the amount of sample available for analysis. Finally, if decalcification is nonessential, this opens the possibility of stable isotope analysis from growth layers of teeth from species whose teeth were previously thought too small to gather enough dentin for adequate analyses. Since inert tissues such as teeth and bones can accumulate long-term stable isotope data that illuminate foraging ecology and habitat use of marine mammals that are typically cryptic and difficult to study, a full understanding of how these samples can best be utilized is important for ecologists reconstructing marine mammal foraging and habitat use patterns.

## Methods

### Stable isotope analysis

I collected teeth (n = 23) from seven marine mammal species stored in archived collections at the National Oceanic and Atmospheric Administration's National Marine Fisheries Service Marine Mammal Laboratory at the Alaska Fisheries Science Center in Seattle, WA and their Southwest Fisheries Science Center in La Jolla, CA, and the Smithsonian National Museum of Natural History in Washington, DC. The seven species were beluga whales (*Delphinapterus leucas*) (n=6), bottlenose dolphins (*Tursiops truncatus*) (n=3), California sea lions (*Zalophus californianus*) (n=3), false killer whales (*Pseudorca crassidens*) (n =2), northern fur seals (*Callorhinus ursinus*) (n=3), Steller sea lions (*Eumatopias jubatus*) (n=3), and short-finned pilot whales (*Globicephala macrorhynchus*) (n=3) (Table 1). I bisected each tooth into equal,

longitudinal halves using a diamond blade on an Isomet saw, adhered half the tooth with putty to a microscope slide for stability, then used a hand-drill (Black and Decker) to collect dentin powder from the tooth half. I drilled across all growth layers within the tooth half and collected 25 to 27 mg of tooth powder into 2 ml cryovial tubes (Table 2). I sealed ~1.5 mg of the untreated powder from each tooth into 5x9 mm tin capsules for bulk stable carbon and nitrogen isotope analyses. I reserved the remaining tooth powder for use in the tooth decalcification process.

To decalcify the dentin samples, I added 1 ml of 0.5 M HCL to the remaining ~24 to 26 mg of tooth powder in each 2 ml cryovial, then refrigerated the samples at 4ºC for 24 hours. Following refrigeration, I centrifuged each sample at 5000 rpm for 5 minutes, then aspirated and discarded the supernatant. I rinsed each sample 5 times with Milli-Q, ultrapure water, centrifuging each sample for 5 minutes at 5000 rpm between rinses, and removed the water with a pipette between each rinse. After the final rinse, centrifuge, and excess water removal, I transferred any remaining water and tooth residue (which was the expected purified collagen) into pre-weighed 5x9 mm tin capsules which were then placed into a 96-well plate. I dried the samples in a 60 to 70ºC drying oven for 24 hours for complete evaporation of all remaining water. I then weighed each sample in its 5x9 tin capsule and sealed the capsules. See Table 1 for all pre- and post-decalcification weights of tooth dentin for each sampled individual.

The Stable Isotope Laboratory at the Department of Earth and Marine Sciences, University of California Santa Cruz, analyzed the $\delta^{13}C$ and $\delta^{15}N$ values for all (intact and decalcified) samples using a Carlo Erba CE1108 elemental analyzer interfaced via a ConFlo III device to a Thermo-Electron Delta Plus XP mass spectrometer. I calculated the average precision of these data as the SD of the $\delta^{13}C$ and $\delta^{15}N$ values from a set of standards (acetanilide), and it was 0.05‰ and 0.21‰, respectively.

Statistical Analysis

To compare the stable carbon and nitrogen isotope values of teeth with vs. without decalcification prior to isotope analysis, I generated two samples from each sampled tooth, one decalcified, the other intact. To determine if there is a consistent bias affecting the stable isotope values of marine mammal teeth introduced via the decalcification process, I subsequently subtracted the decalcified stable isotope value from that of the non-decalcified sample for each tooth, and I used this new value as the response variable in subsequent analysis.

To determine if decalcification or marine mammal type (cetacean vs. pinniped) or species affected the outcome of the stable isotope values from marine mammal teeth that were decalcified or left intact, I developed a mixed effect model that included cetacean (yes or no) as a fixed effect, and species as a random effect. In this model, the fitted intercept value provides an estimate of the bias in isotope values resulting from decalcification. Thus, a significant effect of the fixed effect intercept (defined as $p$-value $<0.05$) would indicate that decalcification affects the stable isotope values of the sample. Similarly, significant effects of marine mammal type would indicate that the bias introduced by decalcification differs across marine mammal order (fixed effect of cetaceans) and/or species (random effects).

I hypothesized that the introduction of variance in the stable isotope values due to decalcification may differ between carbon and nitrogen. However, I assumed that the potential introduction of variability in the stable isotope values due to the decalcification procedure is independent of species or marine mammal order (and therefore did not uses these levels for this analysis). To test the above hypothesis, I conducted F-tests for the equality of two variances

(decalcified and non-decalcified) for each element. I calculated the average precision for these data as the SD of the $\delta^{13}C$ and $\delta^{15}N$ values from a set of standards (acetanilide from A. Schimmelmann, Indiana University, see Schimmelmann et al. 2009), and precision was 0.04 ‰ for nitrogen and 0.05 ‰ for carbon.

## Results

There were no significant effects of marine mammal order (Cetacea vs. Pinnipedia; $p$ = 0.2299 for $\delta^{13}C$ and $p$ = 0.8381 for $\delta^{15}N$), species (as indicated by the small variances associated with the random effects of species for $\delta^{13}C$ (0.043) and $\delta^{15}N$ (0)), or the decalcification process ($p$ = 0.6553 for $\delta^{13}C$ and $p$ = 0.9773 for $\delta^{15}N$) on the stable carbon and nitrogen isotope values from marine mammal teeth (Tables 3 and 4).

The range in the $\delta^{13}C$ and $\delta^{15}N$ values from individual teeth measured across all seven marine mammal species (n = 23 teeth) was -16.9 to -12.2‰ and 11.2 to 19.9‰, respectively, for intact samples, and -16.5 to -12.5‰ and 11.3 to 20.0‰, respectively, for decalcified samples (Table 2). The mean (±SD) $\delta^{13}C$ and $\delta^{15}N$ values across all species for intact and decalcified samples were -14.1±1.3‰ and -14.2±1.2‰ and 16.6±2.4‰ and 16.7±2.3‰, respectively (Table 2, Figure 2). The C:N ratios were slightly higher for intact (3.0±0.2) vs. decalcified (2.8±0.1) teeth (paired t-tests, t = 7.47, df = 22, P < 0.00001) (Table 2).

## Discussion

I found no significant differences in the $\delta^{13}C$ and $\delta^{15}N$ values from paired intact vs. decalcified marine mammal tooth samples and no effects of marine mammal order or species on the results. Brault et al. 2014 also found no differences in the bulk stable isotope values from 10

samples pulled from one sperm whale tooth and underscore their cautious recommendation that decalcification may be unnecessary for the isolation of tooth collagen before stable isotope analysis. My analyses also found no effects of marine mammal order or species on the stable isotope values from decalcified vs. intact teeth indicating that decalcification is unnecessary for carnivorous marine mammals, regardless of species or order.

The amino acids in proteins have different stable isotope values (e.g. Popp et al. 2007) and the process of decalcification could have caused certain proteins to be lost, thereby altering the protein and amino acid content, and therefore the $\delta^{13}C$ and $\delta^{15}N$ values, of the processed vs. intact tooth samples. As Brault et al (2014) point out, water-soluble proteins could be removed in the acid treatment to isolate collagen, whereas the non-water-soluble proteins would remain in the untreated samples, and this could have caused isotopic variation between treatments. However, any protein loss that may have occurred was insufficient to cause changes in the $\delta^{13}C$ or $\delta^{15}N$ values from the teeth.

I found a very slight, but significant, difference in the C:N ratios between treated and intact samples, the magnitude (0.2 ‰ higher for raw samples) and direction of which were the same as those found in Brault et al. 2014. Their explanation of this slight variation is that mineral apatite in teeth contains carbonate (a non-protein source of carbon) that has a slightly higher $\delta^{13}C$ value (~2.0‰) than the carbon in collagen. Given the concentrations of protein and carbonate within intact tooth samples, this higher $\delta^{13}C$ values in apatite should add only a very tiny amount (~0.1‰ or less) to the overall $\delta^{13}C$ values from intact teeth and should therefore not be a significant source of variation for the $\delta^{13}C$ values, but can be observed in the slightly different C:N ratios. Finally, we did not consider lipid to be a contaminant for these samples as all C:N ratios were at or below the expected threshold (3.0) for animal tissues with very low to no lipids.

My data indicate that decalcification is unnecessary for the accurate bulk stable isotope analysis of carnivorous marine mammal teeth. As marine mammal teeth are a commonly archived tissue stored in museums and other specimen collections, they can be targeted for bulk stable isotope analysis to further reconstruct past animal dietary and movement patterns as well as illuminate other ecological and oceanographic processes over time. In addition, without the need for decalcification, the processing of teeth for isotope analysis will be much faster and the amount of material available for analysis will be larger, both of which are useful for maximizing efficiency and the utility of each sample.

FIGURES

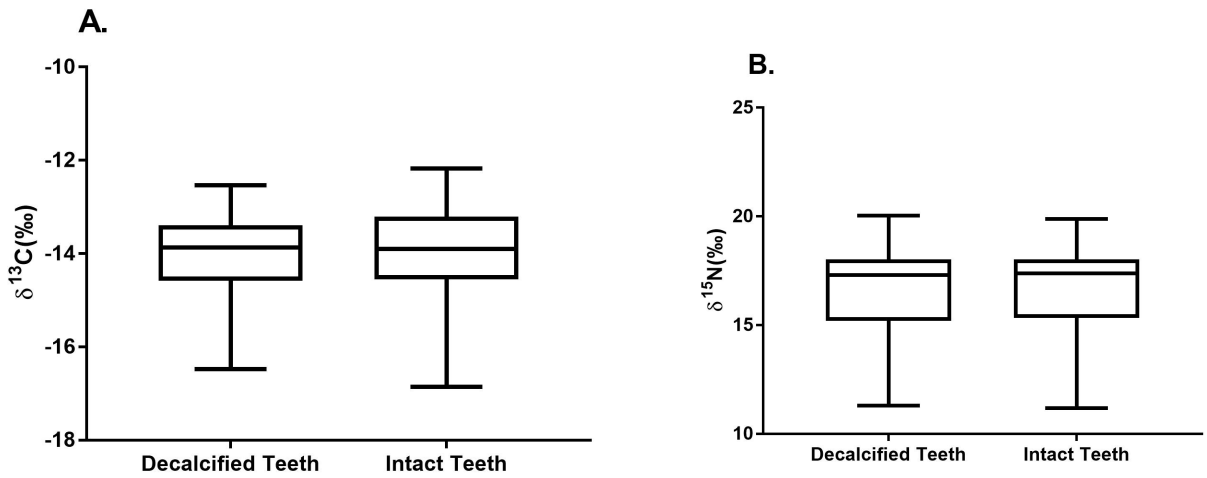


**Figure 1.** The bulk stable A) carbon ($\delta^{13}$C) and B) nitrogen ($\delta^{15}$N) isotope values (‰) from decalcified and raw, untreated (intact) dentin from marine mammal teeth (n = 23 teeth from 7 species) were not significantly different indicating that the decalcification process used to remove minerals from teeth and isolate collagen is not needed for preparation of tooth dentin for bulk stable isotope analysis. Boxes indicate median, and upper and lower 75th and 25$^{th}$ percentiles, and whiskers represent 10$^{th}$ and 90$^{th}$ percentiles.


**Figure 2.** Bottlenose dolphin tooth to scale.


**Figure 3.** Northern Fur Seal tooth to scale.


**Figure 4.** California Sea Lion tooth to scale.

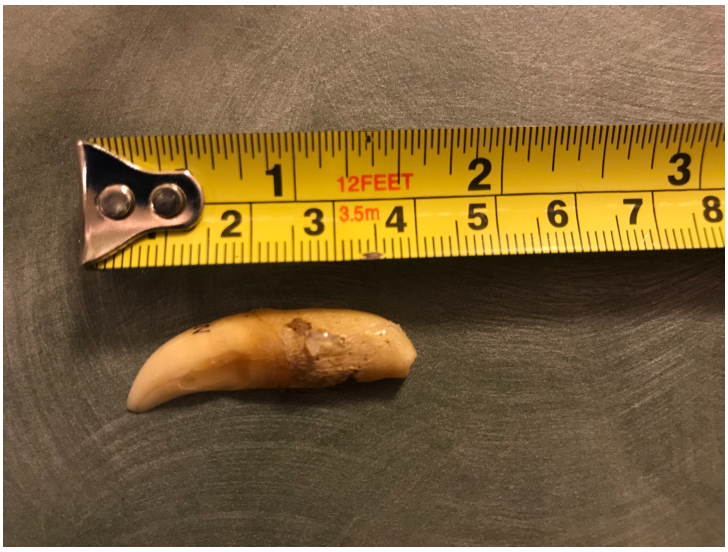


**Figure 5.** Short-finned Pilot Whale tooth to scale.


**Figure 6**. Stellar Sea Lion tooth to scale.


**Figure 7.** False Killer Whale tooth to scale.


**Figure 8.** Beluga Whale tooth to scale.

TABLES

**Table 1.** Table of tooth dentin weights before and after the decalcification process.

| Sample ID | Dentin weight before decalcification (mg) | Dentin weight after decalcification (mg) |
|---|---|---|
| Northern Fur Seal | 25 | 1.9 |
| Northern Fur Seal | 25 | 2.1 |
| Northern Fur Seal | 26 | 1.6 |
| California Sea Lion | 24 | 1.3 |
| California Sea Lion | 25 | 1.1 |
| Stellar Sea Lion | 30 | 1.1 |
| Stellar Sea Lion | 24 | 2.0 |
| Stellar Sea Lion | 25 | 1.5 |
| Bottlenose Dolphin | 26 | 2.2 |
| Bottlenose Dolphin | 28 | 2.7 |
| Bottlenose Dolphin | 27 | 2.6 |
| Shortfin Pilot Whale | 25 | 1.1 |
| Shortfin Pilot Whale | 26 | 1.9 |
| Shortfin Pilot Whale | 27 | 1.6 |
| False Killer Whale | 27 | 1.7 |
| False Killer Whale | 23 | 1.8 |
| Beluga Whale | 49.2 | 36.1 |
| Beluga Whale | 50.4 | 41.4 |
| Beluga Whale | 49.4 | 42.4 |
| Beluga Whale | 51.7 | 38.7 |
| Beluga Whale | 51.1 | 41.8 |
| Beluga Whale | 51.0 | 40.5 |

**Table 2.** The individual and mean (±SD) stable carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) isotope values (‰) and C:N ratios from tooth dentin sampled from seven species of marine mammals (n = 23 teeth). Values reported are from untreated (intact) and decalcified dentin.

| | Intact Dentin | | | Decalcified Dentin | | |
|---|---|---|---|---|---|---|
| Species | $\delta^{13}C$ | $\delta^{15}N$ | C:N | $\delta^{13}C$ | $\delta^{15}N$ | C:N |
| Bottlenose dolphin | -15.5 | 11.3 | 3.1 | -15.8 | 11.4 | 2.8 |
| Bottlenose dolphin | -13.1 | 15.3 | 3.2 | -13.1 | 12.4 | 2.8 |
| Bottlenose dolphin | -13.5 | 17.4 | 3.2 | -14.0 | 17.8 | 2.9 |
| **Species Mean** | **-14.0±1.3** | **14.7±3.1** | **3.2±0.1** | **-14.3±1.4** | **13.9±3.4** | **2.8±0.1** |
| Shortfin Pilot Whale | -13.0 | 17.0 | 3.1 | -13.5 | 17.3 | 2.8 |
| Shortfin Pilot Whale | -13.4 | 16.5 | 3.4 | -13.6 | 17.7 | 2.8 |
| Shortfin Pilot Whale | -13.0 | 17.4 | 3.2 | -14.0 | 16.9 | 2.8 |
| **Species Mean** | **-13.1±0.2** | **17.0±0.4** | **3.2±0.2** | **-13.7±0.2** | **17.3±0.4** | **2.8±0.0** |
| Northern Fur Seal | -16.9 | 14.4 | 3.1 | -16.5 | 16.7 | 2.8 |
| Northern Fur Seal | -16.6 | 16.7 | 2.9 | -16.5 | 14.7 | 2.8 |
| Northern Fur Seal | -16.4 | 16.4 | 2.9 | -16.3 | 16.3 | 2.7 |
| **Species Mean** | **-16.6±0.3** | **15.8±1.3** | **3.0±0.1** | **-16.4±0.1** | **16.0±1.0** | **2.8±0.1** |
| California Sea Lion | -13.2 | 18.0 | 3.1 | -13.3 | 18.1 | 2.8 |
| California Sea Lion | -13.5 | 17.6 | 2.9 | -13.5 | 17.9 | 2.8 |
| California Sea Lion | -13.5 | 18.2 | 2.9 | -13.4 | 18.1 | 2.8 |
| **Species Mean** | **-13.4±0.2** | **18.0±0.3** | **3.0±0.1** | **-13.4±0.1** | **18.0±0.1** | **2.8±0.0** |
| Stellar Sea Lion | -13.4 | 17.5 | 2.8 | -13.3 | 17.6 | 2.8 |
| Stellar Sea Lion | -14.0 | 19.9 | 2.8 | -13.9 | 20.0 | 2.8 |
| Stellar Sea Lion | -15.9 | 15.0 | 2.9 | -15.9 | 15.2 | 2.8 |
| **Species Mean** | **-14.4±1.3** | **17.5±2.5** | **3.0±0.1** | **-14.4±1.3** | **17.6±2.4** | **2.8±0.0** |
| False Killer Whale | -12.9 | 11.2 | 3.4 | -12.5 | 11.3 | 2.8 |
| False Killer Whale | -12.2 | 12.6 | 3.2 | -12.6 | 12.6 | 2.9 |
| **Species Mean** | **-12.5±0.5** | **11.9±1.0** | **2.9±0.1** | **-12.6±0.0** | **12.0±1.0** | **2.9±0.1** |
| Beluga Whale | -14.0 | 19.2 | 2.9 | -13.5 | 17.1 | 2.8 |
| Beluga Whale | -14.1 | 19.0 | 2.9 | -14.0 | 17.1 | 2.8 |
| Beluga Whale | -13.9 | 17.8 | 3.0 | -13.8 | 19.0 | 2.7 |
| Beluga Whale | -14.2 | 17.1 | 3.0 | -14.0 | 19.1 | 2.7 |
| Beluga Whale | -14.6 | 18.0 | 3.0 | -14.6 | 18.0 | 2.7 |
| Beluga Whale | -14.4 | 18.1 | 3.0 | -14.5 | 18.0 | 2.7 |
| **Species Mean** | **-14.2±0.2** | **18.2±0.8** | **3.0±0.1** | **-14.1±0.4** | **18.1±0.9** | **2.7±0.1** |
| **Overall Mean** | **-14.1±1.3** | **16.6±2.3** | **3.0±0.2** | **-14.2±1.2** | **16.7±2.3** | **2.8±0.1** |

**Table 3.** Results of the C data mixed model, including fixed effect of mammal class (cetacean yes or no) and random effects of species.

## Fit Statistics

| | |
|---|---|
| -2 Residual Log Likelihood | 12.436325 |
| -2 Log Likelihood | 6.6981994 |
| AICc | 16.920422 |
| BIC | 19.240176 |

## Random Effects Covariance Parameter Estimates

| Covariance Parameter | Estimate | Std Error | 95% Lower | 95% Upper |
|---|---|---|---|---|
| spp | 0.0431677 | 0.0380974 | 0.0130025 | 0.8516743 |
| Residual | 0.0594177 | 0.0207162 | 0.0331881 | 0.1357072 |

## Fixed Effects Parameter Estimates

| Term | Estimate | Std Error | DFDen | t Ratio | Prob>\|t\| | 95% Lower | 95% Upper |
|---|---|---|---|---|---|---|---|
| Intercept | 0.0452347 | 0.0958255 | 5.4 | 0.47 | 0.6553 | -0.195449 | 0.2859182 |
| cetacean[n] | -0.129614 | 0.0958255 | 5.4 | -1.35 | 0.2299 | -0.370297 | 0.1110697 |

## Random Coefficients

### spp

| spp | Intercept |
|---|---|
| Beluga Whale | -0.228985 |
| Bottlenose Dolphin | 0.0335342 |
| California Sea Lion | 0.0534055 |
| False Killerwhale | -0.084904 |
| Shortfin Pilot whale | 0.2803547 |
| Steller Sea Lion | 0.0235665 |
| Sub-adult male Northern fur seal | -0.076972 |

#### Covariance Matrix

| Random Effect | Intercept |
|---|---|
| Intercept | 0.043168 |

## Fixed Effects Tests

| Source | Nparm | DFNum | DFDen | F Ratio | Prob > F |
|---|---|---|---|---|---|
| cetacean | 1 | 1 | 5.4 | 1.8295307 | 0.2299 |

**Table 4.** Results of the N data mixed model, including fixed effect of mammal class (cetacean yes or no) and random effects of species.

## Fit Statistics

| | |
|---|---|
| -2 Residual Log Likelihood | 72.620955 |
| -2 Log Likelihood | 72.51079 |
| AICc | 86.040201 |
| BIC | 88.188261 |

## Random Effects Covariance Parameter Estimates

| Covariance Parameter | Estimate | Std Error | 95% Lower | 95% Upper |
|---|---|---|---|---|
| spp | 0 | 0 | 0 | 0 |
| Residual | 1.5608035 | 0.4935694 | 0.9135624 | 3.2548008 |

## Fixed Effects Parameter Estimates

| Term | Estimate | Std Error | DFDen | t Ratio | Prob>\|t\| | 95% Lower | 95% Upper |
|---|---|---|---|---|---|---|---|
| Intercept | -0.01305 | 0.4539075 | 20.0 | -0.03 | 0.9773 | -0.959884 | 0.9337847 |
| cetacean[n] | -0.106331 | 0.5136531 | 20.0 | -0.21 | 0.8381 | -1.177792 | 0.965131 |
| cetacean[y] | 0.1846026 | 0.4960369 | 20.0 | 0.37 | 0.7137 | -0.850112 | 1.2193174 |

## Random Coefficients

### spp

| spp | Intercept |
|---|---|
| Beluga Whale | 0 |
| Bottlenose Dolphin | 0 |
| California Sea Lion | 0 |
| False Killerwhale | 0 |
| Shortfin Pilot whale | 0 |
| Steller Sea Lion | 0 |
| Sub-adult male Northern fur seal | 0 |

#### Covariance Matrix

| Random Effect | Intercept |
|---|---|
| Intercept | 0 |

## Fixed Effects Tests

| Source | Nparm | DFNum | DFDen | F Ratio | Prob > F |
|---|---|---|---|---|---|
| cetacean | 2 | 2 | 20.0 | 0.1505433 | 0.8612 |

This material is currently being prepared for submission for publication. Groom, Brenna; Turner Tomaszewicz , Cali; Fleming, Alyson; Semmens, Brice; Kurle, Carolyn. The thesis author was the primary investigator/author of this material.

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