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# Notes

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## Stable isotope signal homogeneity and differences between and within pinniped muscle and skin

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Stable isotope analysis (SIA) is often used to examine diet choice and trophic relationships in marine mammals (*e.g.*, see Hobson 1999, Kelly 2000). However, the technique makes a number of largely untested assumptions (Gannes *et al.* 1997). For example, because marine mammal SIA studies typically sample only a small section of tissue (due to logistical or animal welfare considerations), researchers often assume that biopsy to be representative of the whole animal—that is, that the isotopic signal is homogenous within a tissue. Further, there is little standardization among (or within) studies regarding appropriate tissue sampling protocols, which may lead to bias if isotope ratios are sufficiently heterogeneous within tissues. This problem may be greater when biopsies are obtained through remote darting, a nonlethal method of obtaining samples for a variety of marine mammals (Gemmel and Majluf 1997, Todd *et al.* 1997, Kurle and Worthy 2002, Herman *et al.* 2005) as such methods severely limit the ability to accurately select specific target areas. Isotopic composition may differ across the body within the same tissue type due to differential assimilation or catabolization rates.

In addition, while a variety of tissue types can be used to determine stable isotope values, differences in stable isotope value between tissues are not always known.

Muscle and skin are two tissues that are relatively easily sampled and commonly referenced in studies (see Ostrom *et al.* 1992, Hobson *et al.* 1996, Hobson *et al.* 1997, Kurle and Worthy 2002). In particular, skin samples are easily collected by direct or remotely delivered biopsies (Todd 1997, Gendron *et al.* 2001), and thus may represent a useful source for isotopic values, especially as they are often obtained in the course of other studies (Lambertsen *et al.* 1994). However, isotopic differences between skin and muscle within individuals are not well quantified in marine mammals. To our knowledge, only two studies have directly compared within-individual skin and muscle stable isotope values in cetaceans (Abend and Smith 1995, Todd *et al.* 1997), and few studies have quantified tissue-specific differences in pinnipeds per individual (*e.g.*, see Kurle 2002). Thus, more rigorous comparisons are required to determine the relationship between these two tissues on a within-individual basis.

We investigated the homogeneity of $\delta^{13}C$ and $\delta^{15}N$ values in skin and muscle across the body per individual in three pinniped species: Steller sea lions (*Eumetopias jubatus,* $n = 5$, SSL), California sea lions (*Zalophus californianus,* $n = 6$, CSL), and harbor seals (*Phoca vitulina,* $n = 7$, HS). We also assessed if there are consistent carbon and nitrogen isotope differences between these two commonly sampled tissues. With one exception, all tissue samples were collected opportunistically from beach-strewn carcasses, collected either in the vicinity of Vancouver Island, British Columbia, between the months of November and May in 2003 or 2004 (SSL, CSL), or from various locations on the northeast Maine coastline, between the months of May and August, 1997–2000 (HS). Samples from one remaining SSL were taken from an adult female that died in captivity at an Atlantic coast-based facility (Mystic Aquarium and Institute for Exploration, Mystic, CT).

For SSL and CSL, tissue cores of $5 \times 5$ cm were cut down to (and including) the muscle from the right lateral side off the animal at the following locations: neck (S01), axillary (S02), maximal girth (S03), and flank (S04), and dorsally above the pelvic girdle, close to the spine (S05) and hips (S06) (Fig. 1). For HS, samples were similarly taken, except that at the axillary girth two samples, 10 cm left and right of the dorsal midline (S02L and S02R, respectively) were taken and averaged together for the purposes of analysis; no sample was taken at the hip location. Samples were archived frozen at $-70°C$ (SSL, CSL) or $-27°C$ (HS). In some cases not all samples could be taken due to the poor quality of the carcass (see Appendix, Table A1 and A2).

Skin samples were shaved and excised from underlying blubber tissue using a sterilized scalpel. All samples were dried to constant weight, pulverized using mortar and pestle, and lipid-extracted by bathing in an azeotropic mixture of dichloromethane and methanol for 8 h using a mini-soxhlet array. Samples were then further homogenized to a fine dust using a ball-and-capsule amalgamator (Wig-L-Bug, Pike Technologies, Madison, WI). Stable isotope ratio mass spectrometry conducted at the Biogeochemistry and Paleoproteomics Laboratory, Michigan State University (SSL, CSL) and the Institute for Quarternary Studies, University of Maine (HS) was used to assess $\delta^{13}C$ (SSL, CSL only; insufficient samples of appropriate quality prevented $\delta^{13}C$ analysis for HS) and $\delta^{15}N$ ratios by analyzing subsamples of 4–5 mg of homogenized dust in a Carlo-Erba Elemental Analyzer interfaced with a PRISM stable

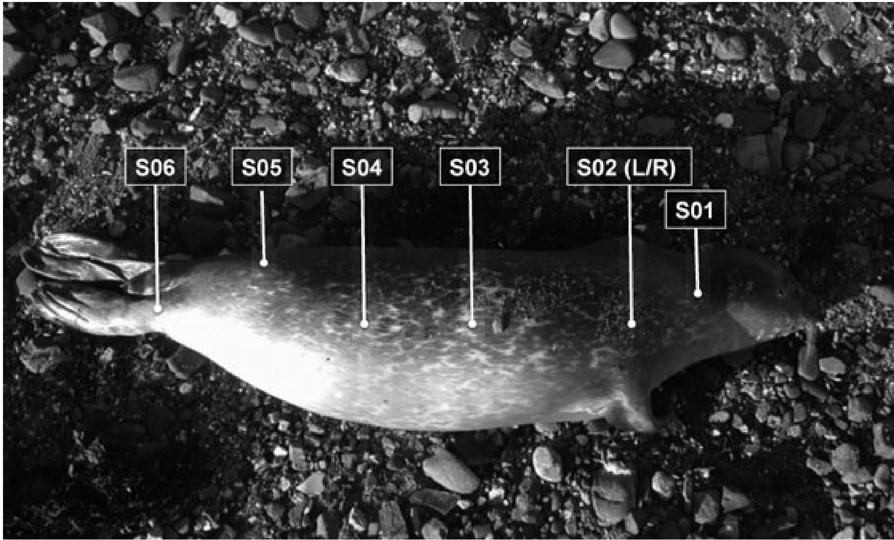


*Figure 1.* Location of biopsied sites. For harbor seals, sites S02L and S02R represent samples taken from the same location on the left- and right-hand sides of the animal, respectively. For otariids, S02 represents a sample taken from the right-hand side only.

isotope ratio mass spectrometer. Stable isotope ratios were expressed as delta values, standardized to Pee Dee Belemnite and atmospheric nitrogen for $\delta^{13}C$ and $\delta^{15}N$ values, respectively, whereby

$$\delta^{y} X(\text{‰}) = \left[\frac{R_{\text{sample}}}{R_{\text{reference}}} - 1\right] \times 10^{3}$$

where $R$ is the ratio of the heavier isotope (of atomic mass $y$) to the lighter form, in either the measured sample or a referenced standard. Procedural reproducibility was estimated to be <0.2‰ for both $\delta^{15}N$ and $\delta^{13}C$ measurements. Species' means, per tissue type, are presented in Table 1.

To examine homogeneity within a specific tissue we used a repeated-measures analysis of variance (ANOVA) that treated individual animals of a species as separate subjects, and different sampling sites as a dependent repeated measure. This analysis demonstrated no statistically significant difference between sample sites in either SSL muscle (ANOVA: $\delta^{13}C$, $F_{5,20} = 0.46$, $P = 0.80$; $\delta^{15}N$, $F_{5,20} = 0.76$ $P = 0.59$) or skin (ANOVA: $\delta^{13}C$, $F_{5,15} = 1.72$, $P = 0.19$; $\delta^{15}N$, $F_{5,15} = 0.97$, $P = 0.47$). Similarly, no significant differences existed between sampled sites from CSL in muscle (ANOVA: $\delta^{13}C$, $F_{5,15} = 0.92$, $P = 0.50$; $\delta^{15}N$, $F_{5,15} = 0.92$, $P = 0.50$). Incomplete sampling series in three animals due to quality of carcasses prevented an analysis of CSL skin $\delta^{13}C$ values across sample sites, although for those sites that were available, values were similar per individual. No differences were found within the skin tissue of harbor seals (ANOVA: $\delta^{15}N$, $F_{5,30} = 0.42$, $P = 0.83$). Figure 2

*Table 1.* Mean ± SD skin and muscle isotope values by species (sample sizes in parentheses).

| Species | Skin | | Muscle | | Skin-muscle | |
|---|---|---|---|---|---|---|
| | $\delta^{13}C$ | $\delta^{15}N$ | $\delta^{13}C$ | $\delta^{15}N$ | $\delta^{13}C$ | $\delta^{15}N$ |
| *E. jubatus*—wild | −14.2 ± 0.5(4) | 17.7 ± 1.1(4) | −15.1 ± 0.2(4) | 17.6 ± 0.5(4) | 0.9 ± 0.4(4) | 0.2 ± 0.9(4) |
| *E. jubatus*—captive | −15.2(1) | 17.4(1) | −16.8(1) | 15.8(1) | 1.6(1) | 1.6(1) |
| *Z. californianus* | −14.8 ± 0.5(4) | 17.8 ± 0.7(4) | −15.4 ± 0.2(5) | 17.2 ± 0.4(5) | 0.6 ± 0.5(3) | 0.5 ± 0.6(3) |
| All otariids, grand mean ± SD | | | | | 0.9 ± 0.5(8) | 0.5 ± 0.8(8) |
| *P. vitulina* | – | 16.5 ± 0.3(6) | – | – | – | – |

Mean discrimination values are calculated on a per individual comparison basis, subtracting muscle from skin values pooled across all biopsy sites.

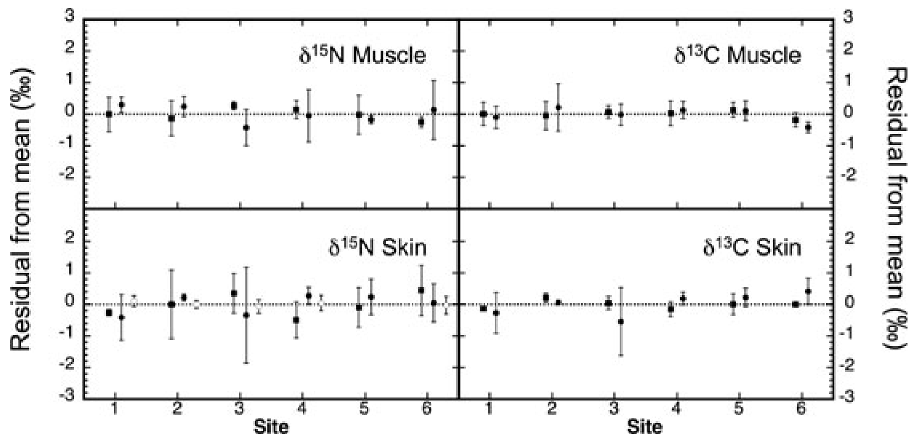


*Figure 2.* Homogeneity of $\delta^{13}C$ and $\delta^{15}N$ isotope signals in *E. jubatus* (solid squares), *Z. californianus* (solid circles), and *P. vitulina* (open circles), as expressed as residuals from the individual mean, averaged across individuals per signal, and tissue type. For *P. vitulina*, site S2 represents an average of S02L and S02R, taken from left- and right-hand sides, respectively. Error bars indicate $\pm 1$ SD. In all cases, no statistical differences exist within a tissue type.

illustrates these results as deviations from the grand mean per individual, averaged per sampling site, per species.

Thus, despite expected differences across the body in physiological processes and biochemical composition, our study found stable isotope signal homogeneity across the body within both muscle and skin, for both carbon and nitrogen isotopes, in all three species. In all cases, variation from the "whole body" mean averaged less than 0.5‰ for both $\delta^{13}C$ and $\delta^{15}N$. This uniformity in stable isotope value is particularly notable given the level of precision of the analysis, and that a change of >3.0‰ in $\delta^{15}N$ is considered ecologically significant as an indication of trophic level change (DeNiro and Epstein 1981, Wada *et al.* 1987, Kurle 2002). Skin nitrogen values for harbor seals were the most homogenous data set, possibly because of consistency in diet. Thus, while variations in the selection of tissue sampling site along a body might be unavoidable, particularly when using remote biopsy techniques (*e.g.*, crossbow, pole-dart, dart projector) on wild, free-ranging animals (Todd 1997, Hoberecht *et al.* 2006), the resultant potential for within-tissue variation in isotope signal appears not to be a primary concern. However, sampling standardization—when possible—is certainly not undesirable; selection of biopsy site should be based on animal safety and logistical considerations. For pinnipeds, we recommend the lower flank of the animal (S04 in our study). This site displayed very little intra-animal variation in our study, and is far removed from most critical areas (*e.g.*, head, abdominal cavity).

As we found that stable isotope values were homogenous within tissues, we collapsed our data within otariid individuals to obtain a single paired skin/muscle value per individual. From these data, isotopic differences between tissues per individual ($\Delta_{\text{skin-muscle}}$) were calculated, and both averaged by individual species, and pooled for both species combined (Table 1). We used paired *t*-tests to determine statistically significant differences between tissues. Our findings were variable; by species,

skin was significantly $^{13}C$ enriched compared to muscle in Steller sea lions (SSL $\Delta_{skin\text{-}muscle}\delta^{13}C$, $t_4 = 5.15, P = 0.01$). It was unclear why this might be, as all other statistical comparisons failed to be significantly different (SSL $\Delta_{skin\text{-}muscle}\delta^{15}N$, $t_4 = 1.00, P = 0.37$; CSL $\Delta_{skin\text{-}muscle}\delta^{15}N$, $t_4 = 2.21, P = 0.11$; CSL $\Delta_{skin\text{-}muscle}\delta^{13}C$, $t_4 = 2.39, P = 0.10$). Pooled across all otariid species, skin tissue was significantly $^{13}C$ enriched when compared to muscle tissue, but the high variability in $\Delta_{skin\text{-}muscle}\delta^{15}N$ values, particularly in SSL, caused skin and muscle tissues to be statistically similar.

Although our study used different species, the level of enrichment found compares favorably with the only other study that examines muscle-skin discrimination in phocids, albeit one that pooled across individuals (Hobson *et al.* 1996). Several studies have compared stable isotope values of different tissue types in marine mammals (Hobson and Clark 1992, Hobson *et al.* 1996, 1997, Lesage *et al.* 2002), but in these cases the lack of per individual tissue comparisons is problematic. Because individual differences may mask differences due to discrimination factors (Gannes *et al.* 1997, Lesage *et al.* 2002), unmatched values (per individual) are less useful in evaluating differences between tissues.

Importantly, the values in this study come from salvaged carcasses. It should be noted that little is known about the effects of decomposition on isotope signals. We note that from our field collection data, the sea lion considered in the highest state of decomposition was isotopically homogenous, but did have greater variation in skin stable isotope value across tissue collection sites. Although not the focus of the current study, a better understanding of the behavior of stable isotopes during carcass decomposition would allow a more rigorous use of the technique with beach-strewn animals.

While our data suggest that muscle and skin samples provide similar isotopic information, we note that currently available processing protocols for skin tissue make this tissue less practical for isotope studies. Primarily, grinding samples to a fine, homogenous dust was easily achieved with muscle tissue, but problematic with skin, introducing additional variation. Skin tissue, once desiccated, takes on a brittle, plastic form that is not easily pulverized, resulting in a dust of uneven particle size. This may result in spurious isotope readings, as indicated in several samples. Processing under cryogenic conditions may help to minimize this problem.

In summary, our results indicate that sufficient homogeneity exists within skin and muscle tissues to suggest that point sampling is indeed representative of entire tissues, and is thus a valid technique in stable isotope studies of marine mammals. This is a particularly salient finding if the sample must be accessed remotely, for example, through biopsy darting. However, we note that a number of other assumptions within SIA still require examination, including a more complete record of across tissue discrimination factors based on per individual comparisons, a need to more accurately assess signal uptake in sampled tissues, as well as signal persistence as a function of tissue metabolic turnover. Furthermore, while this study can serve as a first approximation as to how stable isotope signals behave within marine mammal tissues, more definitive studies are required to determine level of signal homogeneity in cetaceans—particularly as remote delivery biopsy systems are more commonly used with those species.

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## APPENDIX

*Table A1.* Skin stable isotope $\delta^{13}C$ and $\delta^{15}N$ values per individual sampled.

| Species | ID | $\delta^{13}C$ | | | | | | $\delta^{15}N$ | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | S01 | S02 | S03 | S04 | S05 | S06 | S01 | S02 (L/R) | S03 | S04 | S05 | S06 |
| *E. jubatus* | EJ03A | −14.1 | −19.1 | −14.1 | −13.9 | −14.3 | −13.9 | 17.0 | 11.8 | 17.5 | 17.5 | 17.4 | 16.8 |
| | EJ03B | | −14.0 | −14.2 | −14.2 | −13.8 | −14.1 | | 17.0 | 18.3 | 17.5 | 18.3 | 17.9 |
| | EJ04D | −13.8 | −13.2 | −13.6 | −13.6 | −13.8 | −14.4 | 18.6 | 18.8 | 18.6 | 18.8 | 19.4 | 19.8 |
| | EJ04F | −15.5 | −13.6 | −13.7 | −14.3 | −13.3 | −13.8 | 15.7 | 20 | 18.4 | 17.0 | 17.6 | 18.4 |
| | "Stella" | −15.2 | −15.1 | −14.8 | −15.4 | −15.4 | −15.1 | 17.2 | 16.4 | 18.7 | 16.5 | 16.5 | 19.0 |
| *Z. californianus* | ZC03A | −15.4 | | −13.8 | −14.1 | −14.3 | −19.5 | 16.8 | | 19.5 | 17.9 | 17.9 | 12.1 |
| | ZC03B | −14.2 | −14.1 | −14.2 | −14.3 | −14.2 | −14.2 | 18.5 | 18.6 | 18.5 | 18.7 | 18.0 | 17.8 |
| | ZC03C | −14.3 | −14.5 | −15.5 | −14.4 | −14.4 | −14.2 | 17.6 | 17.9 | 16.7 | 18.2 | 18.3 | 17.8 |
| | ZC04A | | | −16.9 | −14.7 | −14.4 | −14.2 | | | 16.5 | 18.9 | 19.3 | 19.1 |
| *P. vitulina* | PV001 | | | | | | | 16.6 | (16.1/16.4) | 16.9 | 16.4 | 16.5 | |
| | PV002 | | | | | | | 16.6 | (16.3/16.5) | 16.6 | 16.6 | 16.6 | |
| | PV003 | | | | | | | 17.1 | (17.1/16.5) | 17.0 | 17.0 | 16.8 | |
| | PV004 | | | | | | | 16.9 | (16.9/17.6) | 16.7 | 16.8 | 16.4 | |
| | PV005 | | | | | | | 16.7 | (16.1/16.2) | 16.0 | 16.2 | 16.7 | |
| | PV006 | | | | | | | 16.2 | (16.6/16.1) | 16.5 | 16.2 | 16.5 | |
| | PV007 | | | | | | | 16.4 | (16.1/16.1) | 16.3 | 16.4 | 16.0 | |

Missing values correspond to insufficient sample to complete that measurement. Note that no $\delta^{13}C$ samples were taken for *P. vitulina*.

*Table A2.* Muscle stable isotope $\delta^{13}C$ and $\delta^{15}N$ values per individual sampled.

| | | $\delta^{13}C$ | | | | | | $\delta^{15}N$ | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Species | ID | S01 | S02 | S03 | S04 | S05 | S06 | S01 | S02 | S03 | S04 | S05 | S06 |
| *E. jubatus* | EJ03A | −15.3 | −15.4 | −15.6 | −15.3 | −15.3 | −15.3 | 16.6 | 17.1 | 17.4 | 17.4 | 16.3 | 16.8 |
| | EJ03B | −15.4 | −15.5 | −15.1 | −14.5 | −14.8 | −15.3 | 17.4 | 17.7 | 18.2 | 17.2 | 18.8 | 18.0 |
| | EJ04D | −14.5 | −14.9 | −14.8 | −15.5 | −15.1 | −15.6 | 17.6 | 17.7 | 17.9 | 18.0 | 17.1 | 17.2 |
| | EJ04F | −15.1 | −14.4 | −14.6 | −14.9 | −14.9 | −14.8 | 18.1 | 18.4 | 18.1 | 17.7 | 17.7 | 17.7 |
| | "Stella" | −16.8 | −17.3 | −16.6 | −16.7 | −16.3 | −16.9 | 16.6 | 14.8 | 16.0 | 15.8 | 16.3 | 15.4 |
| *Z. californianus* | ZC03A | −15.5 | −15.7 | −15.9 | −15.5 | −15.4 | −16.0 | 17.2 | 17.0 | 16.8 | 15.8 | 16.9 | 18.3 |
| | ZC03B | −15.6 | −15.6 | −15.1 | −14.8 | −15.3 | −15.7 | 18.1 | 18.4 | 17.2 | 18.6 | 17.4 | 16.7 |
| | ZC03C | −15.3 | −15.8 | −15.2 | −15.7 | −15.4 | −15.9 | 17.0 | 16.9 | 16.7 | 16.7 | 16.5 | 17.0 |
| | ZC03D | −15.8 | −15.1 | −15.1 | −15.1 | −14.7 | | 17.3 | 17.8 | 17.4 | 16.9 | 17.1 | |
| | ZC03E | −15.8 | −14.2 | −16.2 | −16.1 | −16.1 | −16.4 | 17.8 | 17.1 | 15.8 | 20.5 | 17.1 | 17.2 |

Missing values correspond to insufficient sample to complete that measurement. Note that no muscle samples were taken for *P. vitulina*.