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The influence of lipid-extraction and long-term DMSO preservation on carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) isotope values in cetacean skin

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ABSTRACT

Stable isotope analysis (SIA) has rapidly become a useful tool to study the ecology of wild animal populations, especially for elusive, wide-ranging predators like marine mammals. The development of projectile biopsy techniques resulted in the collection of thousands of cetacean tissue samples that were archived in a dimethyl sulfoxide (DMSO) solution for long-term, multidecadal preservation. Here we examine the influence of DMSO preservation on carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) values by

comparing a set of paired delphinid skin samples stored frozen without preservative and in DMSO for up to 22 yr. Treatment of paired frozen and DMSO-preserved skin in a 2:1 chloroform:methanol solution yielded similar $\delta^{13}C$ and $\delta^{15}N$ values, revealing that DMSO and lipid contamination have similar isotopic effects on skin, and that these effects can be removed using routine lipid-extraction methods. Further, amino acid concentrations in DMSO-preserved and frozen skin tissue were similar, providing independent evidence of minimal protein alteration due to preservation. Access to a rich archive of skin samples preserved in DMSO will expand our ability to examine temporal and spatial variability in the isotope values of cetaceans, which will aid of understanding of how their ecology has been influenced by historical changes in environmental conditions.

Key words: stable isotope analysis, dimethyl sulfoxide, chloroform:methanol, marine mammals, historical ecology, delphinids.

During the past few millennia, marine ecosystems worldwide have experienced an era of unprecedented change (Pauly *et al.* 1998, Jackson *et al.* 2001, Halpern *et al.* 2008, McCauley *et al.* 2015). For the majority of ocean basins, these changes have occurred within the last 200 yr starting with the commercial harvest of top predators in the 19th century (*e.g.*, whaling) followed by the rapid spread of industrial-scale commercial fishing in the 20th century (Frank *et al.* 2005, Myers *et al.* 2007). Ecological data on the abundance, distribution, and natural history of species prior to the latter half of the 20th century are difficult to obtain, but extending time series into the last century is essential for defining a species ecological

plasticity and its ability to adapt to future environmental change. Many studies have used top marine consumers as sentinels of change, either directly *via* fisheries statistics (Pauly *et al.* 1998, Baum *et al.* 2003, Myers and Worm 2003) or indirectly *via* molecular (genetic) or chemical (stable isotope) analysis of their tissues. Molecular- and isotope-based proxies can record shifts in genetic diversity and population structure (Newsome *et al.* 2007*a*, de Bruyn *et al.* 2011, Pinsky *et al.* 2010, Alter *et al.* 2012), dietary preferences and trophic level (Emslie and Patterson 2007, Misarti *et al.* 2009, Szpak *et al.* 2012, Hamilton *et al.* 2014), and movement patterns (Burton *et al.* 2001, Schell 2000).

Stable isotope analysis (SIA) provides ecological data on resource and habitat use that can be directly compared across ancient to contemporary timescales if potential shifts in baseline isotope values can be constrained. Ancient studies are limited to the analysis of collagen or hydroxyapatite in bones or teeth, but historical studies often utilize “soft” tissues archived in museum and research collections by forward-thinking scientists decades and even centuries ago. Such tissues include skin, keratins, muscle, blubber, organs (*e.g.*, liver), and even whole organisms (fish and invertebrates) that are chemically preserved in formalin, ethanol, or dimethyl sulfoxide (DMSO). In a limited number of instances, tissues were preserved frozen for use in some types of genetic analyses (*e.g.*, RNA) that are precluded by use of preservatives.

For marine mammals, especially cetaceans, the expansion of molecular markers to examine population genetic structure and diversity led to the development of projectile biopsy techniques to collect skin and blubber samples from individual animals in

the field (Palsbøll *et al*. 1991). These technical advances gathered momentum in the 1990s, when immersion in DSMO was considered the best preservative for long-term, multidecadal storage (Amos and Hoelzel 1991, Amos 1997). The value of available archives is evident in the genetic literature, which also includes studies to evaluate the influence of preservatives on quantifying molecular markers. Today, many genetic techniques require only small amounts of tissue for analyses. This improved analytical efficiency greatly increases the value of archived tissues for retrospective studies using newer techniques, such as SIA. Several studies have evaluated the effects of DMSO preservation on tissue carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) isotope values (Hobson *et al.* 1997, Todd *et al.* 1997, Barrow *et al.* 2008, Lesage *et al.* 2010). The most recent and comprehensive study concluded that short-term storage (≤1 yr) in DMSO does not significantly impact results when compared to paired samples stored frozen (Lesage *et al.* 2010); however, to fully utilize historic tissue archives requires the assessment of the impact of long-term storage (*i.e.*, decades) in DMSO on the integrity of tissue stable isotope values.

In addition to the effects of chemical preservation on sample integrity, several studies have evaluated the influence of lipid extraction techniques on $\delta^{13}C$ and $\delta^{15}N$ values of squid (Ruiz-Cooley *et al.* 2011), fish (*e.g.*, Murray *et al.* 2006, Ricca *et al.* 2007, Logan and Lutcavage 2008, Mittenbeck *et al.* 2008) and cetacean (Lesage *et al.* 2010, Yurkowski *et al.* 2015) tissues. The effect on carbon isotope values is particularly important, because lipids have $\delta^{13}C$ values that are 4‰–7‰ lower than associated proteins (DeNiro and Epstein 1977, Newsome *et al*. 2010, Cherry *et al*. 2011). More recently, a suite of studies

has reported mixed results on the effects of lipid extraction on $\delta^{15}N$ values. Some show that lipid-extraction results in a significant but small (~1‰) increase in tissue $\delta^{15}N$ values (Pinnegar and Polunin 1999, Sotiropoulos *et al*. 2004, Sweeting *et al*. 2006), while other studies report minimal to no effect of lipid-extraction on tissue $\delta^{15}N$ (Bodin *et al*. 2007, Ricca *et al*. 2007, Barrow *et al*. 2008, Logan and Lutcavage 2008). These studies analyzed different tissues (muscle, liver, whole organisms) and used a wide array of solvents to extract lipids, which may in part contribute to the difference in conclusions. Lastly, some have advocated for the use of normalization equations to correct for the effects of lipid content on tissue $\delta^{13}C$ values (McConnaughey and McRoy 1979, Alexander *et al*. 1996, Fry *et al*. 2003, Post *et al*. 2007), however, these models appear to be sensitive to taxon, tissue type, and extraction technique (*i.e.*, solvent type) such that they cannot be widely applied to taxa for which models do not currently exist.

In this study, we evaluated the effects of DMSO preservation and lipid-extraction on $\delta^{13}C$ and $\delta^{15}N$ in tissue of cetacean (Family Delphinidae) skin samples stored for up to 22 yr in DMSO to determine the long-term impacts of preservation on tissue integrity for use in isotope-based ecological studies. We further tested the specific effects of DMSO preservation on tissue integrity by analyzing amino acid (AA) concentrations in these same tissues through time. Because most consumer tissues analyzed by ecologists are proteinaceous in nature and are often lipid-extracted prior to stable isotope analysis, AA composition following lipid extraction serves as a proxy for tissue integrity due to preservation alone. This study is the first assessment of how multidecadal preservation in DMSO affects

cetacean skin $\delta^{13}C$ and $\delta^{15}N$ values. Characterizing these effects will enable confident use of archived tissue samples for examination of historical shifts in the ecology of marine mammals, which is needed to understand responses to long-term anthropogenic impacts and climate-driven ecosystem change.

Materials and Methods

Sample Collection

Skin samples were collected from individual dolphins sampled as bycatch in fisheries or as strandings between 1991 and 2011. The dolphin specimens selected had skin samples preserved frozen (no preservative or fixative) and in 20% dimethylsulphoxide (DMSO) solution saturated with NaCl at the same time (Amos and Hoelzel 1991, Amos 1997). All samples are archived at the Southwest Fisheries Science Center (SWFSC). Beginning in 1991 and every 4 yr through 2011, 14–16 samples/yr were selected to evaluate the efficacy of lipid extraction techniques and of skin preserved in DMSO for SIA. Total body length and age were used to characterize the life stage class: calf or noncalf of each dolphin selected for the study. Both metrics used standard methodology for small delphinids (*i.e.*, standard total body length, Norris 1961; age determination, Myrick *et al*. 1983).

Stable Isotope Analysis

All skin samples were subsampled to compare extraction techniques. One set of paired frozen and DMSO-preserved samples were lipid-extracted with three consecutive ~24 h soaks in a 2:1 chloroform:methanol solvent solution, then rinsed five times in deionized water and lyophilized (Newsome *et al*. 2006). The other set was simply rinsed in deionized water and lyophilized. This sampling design generated four sets of samples for isotope

analysis: (1) lipid-extracted frozen, (2) lipid-extracted DMSO-preserved, (3) bulk frozen, and (4) bulk DMSO-preserved. Approximately 0.5–0.6 mg of dried skin was weighed into tin capsules and $\delta^{13}C$ and $\delta^{15}N$ values were measured using a Costech 4010 or CarloErba NC2500 elemental analyzer interfaced with a Finnegan Delta Plus XL isotope ratio mass spectrometer (EA-IRMS) at the University of Wyoming Stable Isotope Facility (Laramie, WY). Isotopic results are expressed as δ values, where $\delta^{13}C$ or $\delta^{15}N = 1{,}000 \times (R_{sample} - R_{standard}/R_{standard})$, where R_{sample} and $R_{standard}$ are the $^{13}C/^{12}C$ or $^{15}N/^{14}N$ of the sample and standard, respectively. The internationally accepted standards for $\delta^{13}C$ and $\delta^{15}N$ values are Vienna Pee Dee Belemnite (V-PDB) and atmospheric nitrogen, respectively, and units are parts per thousand, or per mil (‰). Precision for $\delta^{13}C$ and $\delta^{15}N$ was estimated by analysis of internal protein standards; within and among run variation (SD) was ≤0.2‰ for both $\delta^{13}C$ and $\delta^{15}N$. We also analyzed the weight percent carbon and nitrogen concentrations, reported as [C]/[N], of each sample *via* comparison with protein standards with known [C] and [N].

Amino Acid (AA) Concentrations

The large majority of tissues analyzed by ecologists are proteinaceous in nature and preparation protocols include lipid-extraction prior to isotope or AA concentration analysis. Thus, we measured skin AA concentrations of lipid-extracted skin that had been stored frozen or in DMSO preservative as a proxy for tissue integrity. We randomly chose four skin samples from 1991 and five skin samples from 2003 to span the time range encompassed by our sample set. All specimens in this subset were *D. delphis*, and there was one calf in the sample set from the 1991 sample and eight noncalves ranging in age from 11 to 18 yr.

For each of these nine individuals we measured AA concentrations in paired frozen and DMSO-preserved skin samples that had been lipid-extracted, yielding a total of 18 different samples for analysis. Approximately 1.0 mg of skin tissue was hydrolyzed in 6N hydrochloric (HCl) acid at 110°C for 20 h. 100 µL of AA hydrolysate was dried in a centrifugal evaporator and taken back up in 100 µL 20 mM HCl. Samples were sonicated for 5 min and any insoluble material was pelleted by a 5 min spin at full speed in a microcentrifuge. 10 µL of the redissolved samples were combined with borate buffer (100 mM sodium tetraborate, pH 8.8) to a total volume of 80 µL. 20 µL of Waters AccQ-Tag reagent (~10 mM 6-aminoquinolyl-n-hydroxysuccinimidyl carbamate in acetonitrile) was added. The reactions were mixed, incubated at room temperature for 1 min, then at 55°C for 10 min. AA standards were derivatized with the AccQ-Tag reagent in a similar manner and diluted to generate a standard curve of concentrations from 6.25 pmole/µL to 100 pmol/µL in the final reaction volume. Derivatized AAs were analyzed on a Waters Acquity UPLC system. 1 µL aliquots were injected on a Waters AccQ-Tag Ultra C18 column (2.1 × 100 mm) run at 0.7 mL/min at 55°C. AccQ-Tag Eluents A and B are proprietary Waters reagents. From the MSDS, Eluent A, when properly diluted, appears to be an ammonium formate buffer in 0.5% acetonitrile. Eluent B appears to be the same buffer in 60% acetonitrile. Derivatives of AAs were detected at 260 nm in an Acquity system photodiode array detector with a 500 µL flow cell. Peaks were identified, integrated, and quantified with the Waters Empower software package.

Statistical Analysis

We tested a null hypothesis that extraction method, preservative, storage time and life stage had no influence on $\delta^{13}C$ and $\delta^{15}N$ using a linear mixed effects model available in the *nlme* package (Pinheiro *et al*. 2017) of R (v.3.3.2; R Core Team 2017). Storage time is the number of years a sample is stored, which is directly correlated with year of sample collection. Because the focus of our study is to evaluate the influence of time on stable isotope values, we use storage time in our in our analyses rather than year collected. Because a unique set of dolphins was analyzed for each of the six sampling years of our study and each dolphin skin sample received all four possible combinations of extraction method and preservative in each time period, the models included a random effect for individual dolphin nested within sampling period (*i.e.*, sample collection year). This nested random effects structure reflects the study design and potential for interannual variation in the underlying isotopic composition at the base of trophic webs. Akaike's Information Criterion (AIC) was used to compare candidate models, and the model with the lowest AIC was selected as providing the best fit to the data. Additionally, we interpreted support for candidate models using the AIC guidelines in Burnham and Anderson (2002): 0–2 indicates substantial support, 3–7, less support, and >10, no support. We also evaluated the strength of support for each candidate model using ΔAIC, the difference between a candidate model's AIC and the minimum AIC for all candidate models, and Akaike weights, w_i, the ratio of the candidate model's ΔAIC to that for all models. All statistical significance interpretation were made at $\alpha = 0.05$.

A nonparametric Wilcoxon test was used to test the null hypothesis of no difference in AA concentrations between lipid-extracted skin that was stored frozen or preserved in DMSO.

RESULTS

The sample set (n = 90) included 50 males and 40 females, 19 calves, and 71 noncalves from six species: *Delphinus capensis*, *D. delphis*, *Grampus griseus, Lissodelphis borealis, Lagenorhynchus obliquidens*, and *Tursiops truncatus* (Table 1). Age ranged from 0 to 27 yr (n = 67). Calves ranged in age from within days or weeks of birth to 2.5 yr. All samples were processed in 2013, and the difference between the collection date and processing date provided the number of years in storage (*i.e.*, storage time) for analyses.

Skin $\delta^{13}C$ and $\delta^{15}N$ Values

Both frozen and DMSO-preserved skin was not obtained for all samples, thus sample sizes used in statistical analyses ranged from 82 to 89. The relationship in $\delta^{13}C$ and $\delta^{15}N$ values among treatments are illustrated in Figure 1. The summary statistics for isotope values obtained for each sampling period are provided in Table 2 and illustrated in Figure 2.

All linear mixed effects models for both SIA metrics included the nested random effect of sampling period and dolphin within sampling period, and the effect was significant with 0 outside the bounds of the confidence intervals. For $\delta^{13}C$, the model with the lowest AIC included all fixed effects covariates, with interactions, except life stage, and there was no support evident for any of the other candidate models (*i.e.*, $\Delta AIC > 10$). For $\delta^{15}N$, the model with the lowest AIC included only life stage as a fixed effect covariate. The Akaike weight for this model indicated 3.5 times more support than for the only other

candidate model with good support (*i.e.*, ΔAIC < 3), which included preservative, life stage, and their interaction. All other candidate models had less support (Table 3).

The best linear mixed effects model selected for $\delta^{13}C$ indicated that storage time and extraction method significantly influenced the isotopic values obtained. That is, the $\delta^{13}C$ values from bulk extracted skin tissues were significantly lower than those from lipid-extracted skin tissues, and the offset changed with time in storage (years). The change through time reduced the carbon values by <1% (mean = 0.4%, range: 0.1%–0.7%). This difference is negligible, because it is slightly larger than the analytical precision of measuring $\delta^{13}C$ of organic materials *via* EA-IRMS (Fig. 2). Furthermore, there were significant interactions detected. One was for storage time and extraction method indicated that the values obtained differed more through time than expected given the influence of the two factors alone. The other significant interaction detected was for extraction method and preservative type indicating greater variability in $\delta^{13}C$ values from bulk extraction of DMSO preserved skin than expected based on the results for these covariates independently. Thus, we conclude that storage time has a negligible influence on $\delta^{13}C$ values when samples are lipid-extracted prior to isotope analysis, which appears to remove the influence of both lipids and DMSO on skin tissue carbon isotope composition.

For $\delta^{15}N$, the model with the lowest AIC included only life stage as a fixed effect covariate, and the additional evidence from both ΔAIC and w_i metrics support this model as providing the best fit to the data (Table 4, Fig. 1). Thus, revealing no

evidence of extraction method, preservative type or storage time on the data obtained.

Lastly, we found a significant negative linear trend between weight percent [C]/[N] and $\delta^{13}C$ value for bulk skin stored frozen (Fig. 3A, $F = 211.8$, $P < 0.001$, $R^2 = 0.72$), and a significant but noisier linear trend between weight percent [C]/[N] and $\delta^{13}C$ value for bulk skin preserved in DMSO (Fig. 3B, $F = 17.2$, $P < 0.001$, $R^2 = 0.17$).

Amino Acid Concentrations

DMSO preserved samples had slightly lower but not significantly different concentrations for 12 of the 15 AAs in comparison to paired frozen samples. The three AA concentrations that were significantly different between treatments were glycine, threonine, and lysine (Fig. 4). The nonessential AA glycine was significantly higher in DMSO-preserved *vs.* frozen skin ($\chi^2 = 4.79$, $P = 0.03$). In contrast, the other two essential AAswere significantly higher in frozen *vs.* DMSO-preserved skin: threonine ($\chi^2 = 5.60$, $P = 0.02$) and lysine ($\chi^2 = 7.72$, $P = 0.006$).

Discussion

Effects of Storage Time in DMSO on Skin $\delta^{13}C$ and $\delta^{15}N$ Values

Our results show that long-term preservation in DMSO has a negligible impact on cetacean skin $\delta^{13}C$ and $\delta^{15}N$ values when lipid extraction methods are used prior to isotopic analysis (Tables 2, 4). The model selected for $\delta^{13}C$ also revealed that bulk skin tissues that were not lipid-extracted were significantly lower by an average of 1.8‰ (CI: −2.1‰ to −1.5‰, Table 4) than those from lipid-extracted skin tissues. As expected, extraction method, preservative, and storage time had no influence on skin $\delta^{15}N$ values but life stage class did (Tables 2, 4). The possible

influence of sample collection year, which is directly correlated with storage time, and life stage on $\delta^{13}C$ and $\delta^{15}N$ values is well documented in the literature (*e.g.*, Schell 2000, Newsome *et al*. 2006, 2007*b*). Our study encompassed the variability associated with these characteristics that are at the core of SIA ecological studies (Table 2, Fig. 2), providing the necessary framework to support our conclusions that storage time and preservative are not an issue for cetacean studies when skin tissue is lipid-extracted prior to analysis.

Effects of DMSO and Lipids on Skin $\delta^{13}C$ Values

Lipid-extraction of DMSO-preserved specimens produced similar $\delta^{13}C$ values as those for lipid-extracted skin preserved frozen (Fig. 1A). This finding demonstrates that both lipids and any residual DMSO can be adequately removed with commonly used lipid-extraction protocols (Bligh and Dyer 1959). Lesage *et al*. (2010) came to a similar conclusion in an experiment using both odontid and balaenopterid skin that was immersed in DMSO for only 1 yr. Our results extend this timeline to >20 yr and confirms that the isotopic integrity of historic cetacean skin samples that have been preserved in DMSO for multiple decades is intact.

Comparison of skin $\delta^{13}C$ values with weight percent carbon and nitrogen concentration data, reported here as [C]/[N], suggest that long-term immersion in DMSO may actually extract lipids that are weakly or moderately bound to skin proteins and accounts for the difference in values obtained from frozen and DMSO preserved skin tissues when not lipid extracted (Fig. 3). DMSO (C_2H_6SO) is a compact organic polar solvent that can bind directly with tissue proteins, carbohydrates, and lipids (Jacob and Herschler 1975, Szmant 1975). While we did not directly

measure the $\delta^{13}C$ value of DMSO, it is likely lower than the lipid-extracted skin that we analyzed (range: −19‰ to −15‰; Fig. 1A); this assumption was also made by previous studies (Lesage *et al*. 2010). Because neither lipids nor DMSO contain nitrogen, lipid and DMSO contamination should increase tissue [C]/[N], and as [C]/[N] increase, tissue $\delta^{13}C$ values are predicted to decrease because both contaminants have lower $\delta^{13}C$ values than skin proteins (DeNiro and Epstein 1977, Cherry *et al*. 2011). This prediction is supported by our data for bulk frozen skin samples that contain lipids (black circles, Fig. 3A), where tissue $\delta^{13}C$ decreases as [C]/[N] increase; note the large range in [C]/[N] (~3.5–6.5) and the tight negative linear relationship (R^2 = 0.72) in Fig. 3A. In comparison, bulk tissues preserved in DMSO have a narrower range in [C]/[N] (~3.5–5.0) and the relationship between $\delta^{13}C$ and [C]/[N] was much noisier (R^2 = 0.17) than that between [C]/[N] and $\delta^{13}C$ for bulk frozen skin tissue (black circles, Fig. 3B). Given its affinity to bind to proteins (Jacob and Herschler 1975, Szmant 1975), we hypothesize that DMSO, a polar solvent, is replacing lipids in skin tissue. Such replacement would explain the decrease in range of [C]/[N] observed in DMSO-preserved skin if complete DMSO saturation yielded a maximum skin [C]/[N] of ~5. Note that pure proteins have a [C]/[N] of ~3.0–3.5 (Ambrose and Norr 1992), which are the ratios observed for lipid-extracted frozen skin (white circles, Fig. 3A, B).

Effects of Lipid-Extraction on Skin $\delta^{15}N$ Values

Our results show that use of (2:1) chloroform:methanol to extract lipids from cetacean skin does not significantly alter skin $\delta^{15}N$ values (Fig. 1B). In contrast, previous studies have shown that lipid-extraction with these solvents significantly

increases the $\delta^{15}N$ values of muscle by ~0.8‰–1.8‰ relative to bulk tissue that was not lipid-extracted (Sotiropoulos *et al.* 2004, Murray *et al.* 2006, Sweeting *et al.* 2006, Bodin *et al.* 2007, Mintenbeck *et al.* 2008, Logan and Lutcavage 2008). Theoretically, polar solvents could extract nonlipid nitrogen-containing cellular components (*e.g.*, amino acids), and previously observed ^{15}N enrichments in lipid-extracted tissues would suggest that leached molecules contain isotopically light nitrogen (^{14}N). Many have suggested that less harsh (*i.e.*, less polar) solvents such as petroleum ether should be used to minimize the leaching of isotopically light nitrogen during lipid-extraction (Degens *et al.* 1968, Schlechtriem *et al.* 2003, Sotiropoulos *et al.* 2004, Søreide *et al.* 2006, Sweeting *et al.* 2006), however, such solvents are not as effective in treating lipid-rich tissues (*e.g.*, liver) of top marine consumers such as cetaceans (Logan and Lutcavage 2008, Ryan *et al.* 2012) and sea turtles (Medeiros *et al.* 2015). We recommend that future studies use a 2:1 chloroform:methanol solvent solution (Bligh and Dyer 1959) to prepare cetacean skin for $\delta^{13}C$ and $\delta^{15}N$ analysis regardless of preservative.

Effects of DMSO Preservation on Skin Amino Acid Concentrations

Despite the small number of samples subjected to amino acid analysis (n = 9 per treatment), three AAs revealed an effect of preservative on their quantitative concentrations (Fig. 4). The nonessential AA glycine ($C_2H_5NO_2$) is the most abundant AA in cetacean skin, and it is the smallest and most simple AA in terms of structure. Immersion in DMSO decreases the solubility of glycine, but only solvent concentrations >40% (Arakawa *et al.* 2007), which is twice the concentration used to preserve the skin samples analyzed in this study. Thus, the only explanation

we have for the observed pattern in glycine concentrations between paired DMSO-preserved and frozen skin is that long-term frozen storage in the absence of a polar preservative like DMSO may denature skin proteins, such that very simple polar AAs like glycine could leach from the sample when it is processed *via* lipid-extraction for stable isotope analysis. Conversely, a possible explanation for why threonine and lysine concentrations were lower in DMSO-preserved versus frozen skin is that DMSO influences cell permeability (Gordeliy *et al*. 1998) such that repeated rinsing with solvents and water during the lipid-extraction process may have preferentially leached these essential AAs. Note that while significant, the overall difference in concentration of these two essential amino acids is small (<0.5%) in comparison to the observed differences in glycine concentrations (~2%) between paired DMSO-preserved and frozen samples. Lipid-extraction with chloroform:methanol could result in preferential leaching of ^{15}N-depleted nitrogenous cell components (Søreide *et al*. 2006), however, both the DMSO-preserved and frozen skin samples were lipid-extracted prior to amino acid analysis. Thus, this hypothesis does not explain the observed differences in AA composition between tissue treatments (Fig. 4).

Summary

Our study is the first to assess multidecadal effects of DMSO preservation on cetacean skin δ^{13}C and δ^{15}N values. Overall, our results show that treatment of cetacean skin with a 2:1 chloroform:methanol solvent solution (Bligh and Dyer 1959) is a reliable method to remove effects on δ^{13}C values associated with both the presence of lipids and long-term (>20 yr) preservation in DMSO. We also found that lipid-extraction with these solvents

had no significant effect on skin $\delta^{15}N$ values. Long-term preservation in DMSO also had subtle impacts on skin [AA], but such effects did not influence skin $\delta^{13}C$ or $\delta^{15}N$ values. Our findings show that cetacean skin preserved in DSMO for multiple decades can be used in isotope-based studies to examine historical shifts in diet, habitat use, and movement patterns if changes in the isotopic composition of the base of the food web can be constrained.

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Figure 1. Relationships between (A) $\delta^{13}C$ and (B) $\delta^{15}N$ values of skin stored frozen that was lipid-extracted (*x*-axis) and those that were preserved in DMSO and lipid-extracted (black circles), stored frozen and not lipid-extracted (white circles), and preserved in DMSO and not lipid-extracted (gray circles). Numbers in parentheses denote sample sizes for each comparison; dashed lines represent a 1:1 relationship.

Figure 2. The difference ($\Delta^{13}C$ or $\Delta^{15}N$) in (A) $\delta^{13}C$ and (B) $\delta^{15}N$ between lipid-extracted skin that was stored frozen and preserved in DMSO for up to 22 yr. Black circles represent mean offsets for each group; error bars represent standard deviation.

Figure 3. Relationship between weight percent carbon-to-nitrogen ratio and $\delta^{13}C$ value of bulk (black circles) and lipid-extracted (white circles) skin tissue stored (A) frozen or (B) preserved in DMSO. Solid black lines are linear regressions for bulk skin (black circles) that was not lipid-extracted prior to isotope analysis.

Figure 4. Relative nonessential (left) and essential (right) amino acid concentrations of lipid-extracted skin that was stored frozen (black bars) or preserved in DMSO (white bars); numbers in parentheses denote sample sizes for each treatment. Asterisks denote significant differences in concentrations between treatments. Included in the analysis are nonessential amino acids alanine (Ala), glycine (Gly), serine (Ser), aspartic acid (Asp), glutamic acid (Glu), arginine (Arg), proline (Pro), tyrosine (Tyr), and essential amino acids leucine (Leu), isoleucine (Ileu), threonine (Thr), valine (Val), phenylalanine (Phe), lysine, (Lys), and histidine (His).

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Table 1. Sample sizes for the six delphinid species used in this study grouped by year of collection.

Year	*Delphinus capensis*	*Delphinus delphis*	*Tursiops truncatus*	*Grampus griseus*	*Lagenorhynchus obliquidens*	*Lagenorhynchus borealis*	Annual totals
1991	–	15	–	–	1	–	16
1995	3	11	–	1	–	–	15
1999	–	14	–	–	–	1	15
2003	3	10	1	1	–	–	15
2007	11	1	1	1	–	–	14
2011	13	–	2	–	–	–	15
Total	30	51	4	3	1	1	90

Table 2. Offset (‰) in $\delta^{13}C$ ($\Delta^{13}C_{FROZEN-DMSO}$) or $\delta^{15}N$ ($\Delta^{15}N_{FROZEN-DMSO}$) values between lipid-extracted and bulk cetacean skin samples preserved frozen and in DMSO grouped by year of collection; error is presented as one standard deviation (SD).

Year	Lipid-extracted		Bulk	
	$\Delta^{13}C_{FROZEN-DMSO}$ Mean ± SD	$\Delta^{15}N_{FROZEN-DMSO}$ Mean ± SD	$\Delta^{13}C_{FROZEN-DMSO}$ Mean ± SD	$\Delta^{15}N_{FROZEN-DMSO}$ Mean ± SD
1991	0.3 ± 0.4	−0.3 ± 0.4	0.6 ± 0.8	0.0 ± 0.4
1995	0.3 ± 0.2	−0.1 ± 0.3	0.1 ± 1.0	0.0 ± 0.8
1999	0.2 ± 0.2	−0.2 ± 0.3	0.3 ± 0.6	0.0 ± 0.2
2003	0.1 ± 0.2	−0.1 ± 0.3	0.1 ± 0.7	−0.1 ± 0.5
2007	0.1 ± 0.2	−0.1 ± 0.3	0.7 ± 1.4	0.1 ± 0.3

2011	0.2 ± 0.3	−0.2 ± 0.3	0.8 ± 0.7	0.0 ± 0.2
Mean ± SD	0.2 ± 0.3	−0.2 ± 0.3	0.4 ± 0.9	0.0 ± 0.4

Table 3. Akaike's Information Criterion (AIC) is presented for each candidate linear mixed effects model fit separately to $\delta^{13}C$ and $\delta^{15}N$; "*" indicates interactions among fixed effect covariates were included, and "+" means no interactions. All models included a random intercept and a nested random effect of dolphin within sampling period; see Methods for additional explanation of experimental design. The models with the lowest AIC are highlighted in bold for $\delta^{13}C$ and $\delta^{15}N$, and the other models with substantial support are italicized. The additional information used to evaluate the strength of support for each candidate model is presented as ΔAIC, the difference between the candidate model's AIC and the minimum AIC, and as Akaike weights, w_i, the ratio of the candidate model's ΔAIC to that for all models.

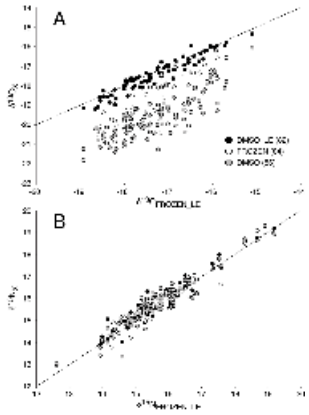
Model formula	df	$\delta^{13}C$			$\delta^{15}N$		
		AIC	ΔAIC	w_i	AIC	ΔAIC	w_i
y ~ 1	4	1,144.2	488.6	0.00	377.2	6.2	0.00
y ~ 1 + Storage Time	5	1,144.3	488.8	0.00	379.1	8.2	0.00
y ~ 1 + Preservative	5	1,147.2	491.7	0.00	373.7	2.7	0.05
y ~ 1 + Extraction Method	5	704.9	49.4	0.00	384.4	13.5	0.00
y ~ 1 + Life Stage	5	1,146.4	490.8	0.00	**371.0**	**0.00**	**0.74**
y ~ 1 + Storage Time * Preservative	7	1,155.1	499.5	0.00	386.4	15.4	0.00
y ~ 1 + Storage Time * Extraction Method	7	679.1	23.5	0.00	397.5	26.5	0.00
y ~ 1 + Storage Time * Life Stage	7	1,152.8	497.2	0.00	380.5	9.5	0.00
y ~ 1 + Preservative * Extraction Method	7	674.1	18.6	0.00	377.4	6.4	0.00
y ~ 1 + Preservative * Life Stage	7	1,151.1	495.6	0.00	*372.2*	*1.3*	*0.21*
y ~ 1 + Extraction Method * Life Stage	7	708.8	53.3	0.00	383.8	12.8	0.00
y ~ 1 + Storage Time * Preservative * Extraction Method	11	**655.5**	**0.000**	**1.00**	410.4	39.4	0.00
y ~ 1 + Storage Time * Preservative * Extraction Method * Life Stage	19	690.0	34.5	0.00	450.9	79.9	0.00

y ~ 1 + Storage Time + Preservative + Extraction Method	7	706.5	51.0	0.00	383.0	12.0	0.00
y ~ 1 + Storage Time + Preservative + Extraction Method + Life Stage	8	708.7	53.1	0.00	377.1	6.2	0.00

Table 4. Summary of fit statistics for the linear mixed effects model for $\delta^{13}C$ and $\delta^{15}N$ identified as the best model by Akaike's Information Criterion. The intercept is the base condition for skin tissue preserved frozen and lipid extracted for calves sampled in 2011 (*i.e.*, the minimum time stored), and each subsequent line is the parameter difference from base. *P*-values < 0.05 are in bold; CI = confidence interval; *T* = Wald test value.

	$\delta^{13}C$				
	Estimate	SE	CI	*T*	*P*
Fixed effect covariates					
Intercept	−16.476	**0.365**	−17.194 to −15.757	−45.163	**<0.001**
Storage time (yr)	−0.068	**0.027**	−0.122 to −0.014	−2.526	**0.013**
DMSO	−0.076	**0.138**	−0.348 to 0.197	−0.547	**0.585**
Bulk (not lipid-extracted)	−1.806	**0.136**	−2.075 to −1.537	−13.236	**<0.001**
Storage time and DMSO	−0.012	**0.010**	−0.032 to 0.009	−1.122	**0.263**
Storage time and bulk	−0.041	**0.010**	−0.061 to −0.021	−4.045	**<0.001**
DMSO and bulk	**0.827**	**0.195**	**0.443 to 1.211**	**4.241**	**<0.001**
Storage time, DMSO, and bulk	−0.015	**0.015**	−0.044 to 0.013	−1.053	**0.294**
Random effects		SD	CI		

Sampling period		0.407	0.186 to 0.894		
Dolphin within sampling period		0.519	0.433 to 0.622		
Residual		0.448	—		
			$\delta^{15}N$		
Fixed effect covariates	Estimate	SE	CI	*T*	*P*
Intercept	16.291	0.336	15.628 to 16.953	48.446	**<0.001**
Noncalf	−0.667	0.212	−1.088 to −0.245	−3.147	**0.002**
Random effects		SD	CI		
Sampling period		0.687	0.35 to 1.346		
Dolphin within sampling period		0.761	0.651 to 0.89		
Residual		0.250	—		



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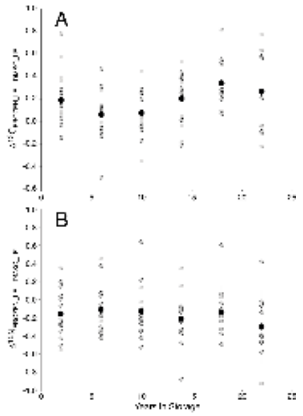

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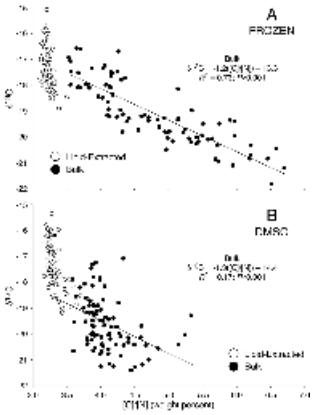

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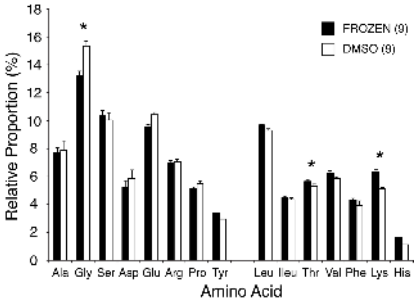

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