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Effect of ethanol preservation on stable carbon and nitrogen isotope values in cetacean epidermis: Implication for using archived biopsy samples

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Stable isotope analyses of C and N ($\delta^{13}C$ and $\delta^{15}N$) provide an integrative way to assess dietary and habitat preferences of marine organisms. This approach has been increasingly used over the last decade to investigate these questions in apex marine predators such as marine mammals (*e.g.,* Lesage *et al.* 2001, Das *et al.* 2003, Zhao *et al.* 2004, Pinela *et al.* 2010). A variety of tissues have been used for stable isotope analyses, including remotely collected skin and blubber biopsy samples from free-ranging individuals (*e.g.,* Hooker *et al*. 2001, Kiszka *et al.* 2011). Ethanol or DMSO are often used as preservative to store samples, especially for logistical constraints in the field, and given the primary focus of most studies using biopsy samples has been mostly genetic analyses. With the recent advances in analytical technologies, which allow for using small amounts of material, biopsy samples have been increasingly used to investigate a variety of other questions, including ecological ones using chemical tracers such as stable isotopes.

The use of preservatives, as well as lipid extraction and normalization, are sources of biases and should be accounted for in order to avoid misinterpretation of stable isotope data (*e.g.,* McConnaughey and McRoy 1979, Hobson *et al.* 1997, Post *et al*. 2007, Logan *et al.* 2008, Lesage *et al.* 2010). Accurately predicting errors related to preservation would enable the use of archived samples, such as biopsies, in studies of trophic ecology and habitat use of marine mammals, including diet estimations using isotopic mixing models. Lesage *et al.* (2010) assessed whether lipid extraction of DMSO-preserved samples could provide equivalent estimates of isotopic signatures

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to those from unpreserved samples. Effects were determined to be predictable and consistent across four cetacean species groups with different epidermis structure and thickness (Lesage *et al.* 2010). Ethanol (EtOH) is commonly used to store cetacean biopsy samples, but effects of this preservative on isotopic signatures of epidermis remains undocumented. EtOH storage may affect stable isotope signatures in other taxa and tissue, but the magnitude and direction of change is inconsistent among studies (Hobson *et al*. 1997, Gloutney and Hobson 1998, Kaehler and Pakhomov 2001, Sakarinos *et al*. 2002, Sweeting *et al*. 2004, Kelly *et al*. 2006, Barrow *et al.* 2008, Syväranta *et al*. 2008). For instance, while EtOH preservation depleted $\delta^{13}C$ values in *Drosophila* tissues (Ponsard and Amlou 1999), EtOH preservation had a positive effect on $\delta^{13}C$ values in fish, clam, octopus (muscle), and kelp samples (Kaehler and Pakhomov 2001, Sarakinos *et al.* 2002). The effect of EtOH on $\delta^{15}N$ values is generally positive, although magnitude varies between studies and organisms (*e.g.,* Sarakinos *et al*. 2002, Kelly *et al*. 2006, Bugoni *et al*. 2008, Carabel *et al*. 2009). When observed, the increase in $\delta^{13}C$ values during storage has been attributed to the loss of lipids, which are depleted in ^{13}C relative to proteins and carbohydrates. However lipids most often have to be removed to avoid misinterpretations of the C isotopic signature in trophic studies, especially in soft tissues of organisms (De Niro and Epstein 1978, Tieszen *et al*. 1983). Overall, effects of preservatives (including EtOH) are relatively inconsistent among taxa and tissue. It clearly highlights the need for species- and tissue-specific experiments.

Here, we address the question of the impact of cetacean skin sample preservation in EtOH on isotopic signatures. We used the epidermis from 16 common dolphins (*Delphinus delphis*) and compared stable carbon and nitrogen isotope values of tissue aliquots immersed in a solution of EtOH (70% ethanol v/v) to aliquots from the same individuals, but preserved frozen (–20°C), a technique with no effect on isotopic signatures (Sweeting *et al*. 2004). Tissues were collected from well-preserved to moderately decomposed carcasses (codes 2–3, *i.e.*, little scavenger damage, fresh to mild odor, blubber firm to slightly oily, wrinkled to cracked skin with little to no abrasion, intact organs; Geraci and Lounsbury 2005) found dead along the French Atlantic coast from 2001 to 2009. Tissues were preserved in EtOH and frozen for periods varying between 11 and 108 mo. The ethanol was evaporated at 45°C over 48 h, and the samples were ground to a powder and freeze-dried to constant mass. Lipids from ethanol-preserved and frozen epidermis samples were removed by two successive extractions (1 h shaking in cyclohexane at room temperature and subsequent centrifugation) prior to analysis. After drying, small subsamples (0.35–0.45 mg $\pm$ 0.001 mg) were prepared for analysis. Stable isotope measurements were performed with a continuous-flow isotope-ratio mass spectrometer (Delta V Advantage, Thermo Scientific, Germany) coupled to an elemental analyzer (Flash EA1112, Thermo Scientific, Italy). Reference gas was calibrated against International Reference Materials (IAEA-N1, IAEA-N2, and IAEA-N3 for nitrogen; NBS-21, USGS-24, and IAEA-C6 for carbon). Results are expressed in the d notation relative to Vienna PeeDee Belemnite and atmospheric N_2 for $\delta^{13}C$ and $\delta^{15}N$, respectively, according to the equation: $\delta X = [(R_{sample}/R_{standard}) - 1] \times 1{,}000$, where X is ^{13}C or ^{15}N and R is the isotope ratio ^{13}C:^{12}C or ^{15}N:^{14}N, respectively. Replicate measurements of a laboratory standard (acetanilide) indicated that analytical errors were $<0.1‰$ for $\delta^{13}C$ and $\delta^{15}N$. Percent C and N elemental composition of tissues were used to calculate the sample C:N ratio, a metric which indicates efficient lipid removal from cetacean epidermis when values are <4 (Lesage *et al*. 2010; Table 1).

Table 1. Isotopic signatures from ethanol-preserved *vs*. frozen common dolphin (*Delphinus delphis*) lipid-extracted epidermis samples.

Year	Sex	Size (cm)	Ethanol-preserved $\delta^{13}C$	Ethanol-preserved $\delta^{15}N$	Frozen $\delta^{13}C$	Frozen $\delta^{15}N$	C:N	Time preserved (months)
2002	Male	213	–18.1	11.9	–18.6	11.9	3.88	108
2002	Male	175	–18.0	12.7	–17.1	12.3	3.24	108
2002	Male	193	–17.1	13.2	–15.8	14.5	3.60	108
2005	Male	177	–18.4	13.0	–18.1	13.1	3.52	76
2005	Female	198	–17.3	14.1	–16.5	14.1	3.56	76
2005	Female	197	–17.6	14.2	–17.0	14.0	3.32	76
2005	Female	184	–16.9	13.8	–17.6	13.8	3.20	76
2006	Male	200	–18.0	12.6	–17.8	12.4	3.32	48
2006	Male	208	–18.8	11.8	–18.0	11.7	3.67	48
2009	Male	210	–18.2	12.5	–18.1	12.5	3.38	14
2009	Male	218	–19.1	12.3	–18.2	12.3	3.28	14
2009	Male	183	–18.3	13.1	–17.7	12.8	3.12	14
2009	Male	201	–18.3	13.0	–17.6	12.9	3.23	14
2009	Male	195	–18.5	12.9	–18.2	12.7	3.41	14
2009	Male	187	–18.5	13.4	–17.7	13.3	3.55	11
2009	Male	213	–18.0	13.1	–17.8	12.6	3.32	11

Storage duration in EtOH had no effect on $\delta^{13}C$ or $\delta^{15}N$ values (r_p for frozen *vs*. EtOH preserved samples = 0.10 and 0.20, respectively). Epidermis treated with EtOH and lipid-extracted had $\delta^{13}C$ values depleted relative to frozen lipid-extracted samples (paired *t*-tests: df = 15, $P = 0.002$). Depletion was observed in all but two EtOH-preserved samples, resulting in an average depletion of –0.48‰ relative to $\delta^{13}C$ of frozen samples, with a high variability about the mean (SD = 0.51‰, range = –1.31‰ to 0.64‰) (Table 1). The use of a linear regression did not help explain the variability in the data, as the relationship had a poor overall fit ($R^2_{adj} = 0.46$, mean errors in $\delta^{13}C$ values of ± 0.42‰, with a maximum error (95% CL) of ± 0.98‰), with an intercept not different from zero ($P = 0.56$) and a slope, not different from one ($P = 0.49$) (Fig. 1). In contrast, there appeared to be no significant effect of EtOH on $\delta^{15}N$ values once tissues were lipid-extracted (paired *t*-tests: df = 15, $P = 0.624$), with a mean enrichment of +0.05‰ for EtOH-treated over frozen samples (SD = 0.38‰, range = –1.25‰ to 0.39‰). This trend was confirmed when regressing $\delta^{15}N$ values of frozen samples against those of EtOH-preserved tissue, as the relationship was highly predictable and associated with a good overall fit ($\delta^{15}N_{Frozen} = -0.53510 + 1.1465 \times \delta^{15}N_{EtOH}$; $R^2_{adj} = 0.76$, $P < 0.0001$), with a slope not significantly different from one ($P = 0.80$; Fig. 2). Mean and maximum errors (95% CL) associated with the regressed $\delta^{15}N$ values were ±0.22‰ and 0.74‰, respectively. Preservation in EtOH had no effect on sample variance for $\delta^{13}C$ or $\delta^{15}N$ values (Levene's *F*, $P = 0.56$ and 0.47, respectively).

The effect of preservatives (*e.g.,* formalin, DMSO, freezing, EtOH, and formalin-EtOH) on $\delta^{13}C$ and $\delta^{15}N$ isotopic signatures has been investigated for many taxa (including invertebrates and vertebrates) and tissues (*e.g.,* Mullin *et al.* 1984, Hobson *et al.* 1997, Todd *et al.* 1997, Ponsard and Amlou 1999, Sweeting *et al*. 2004,

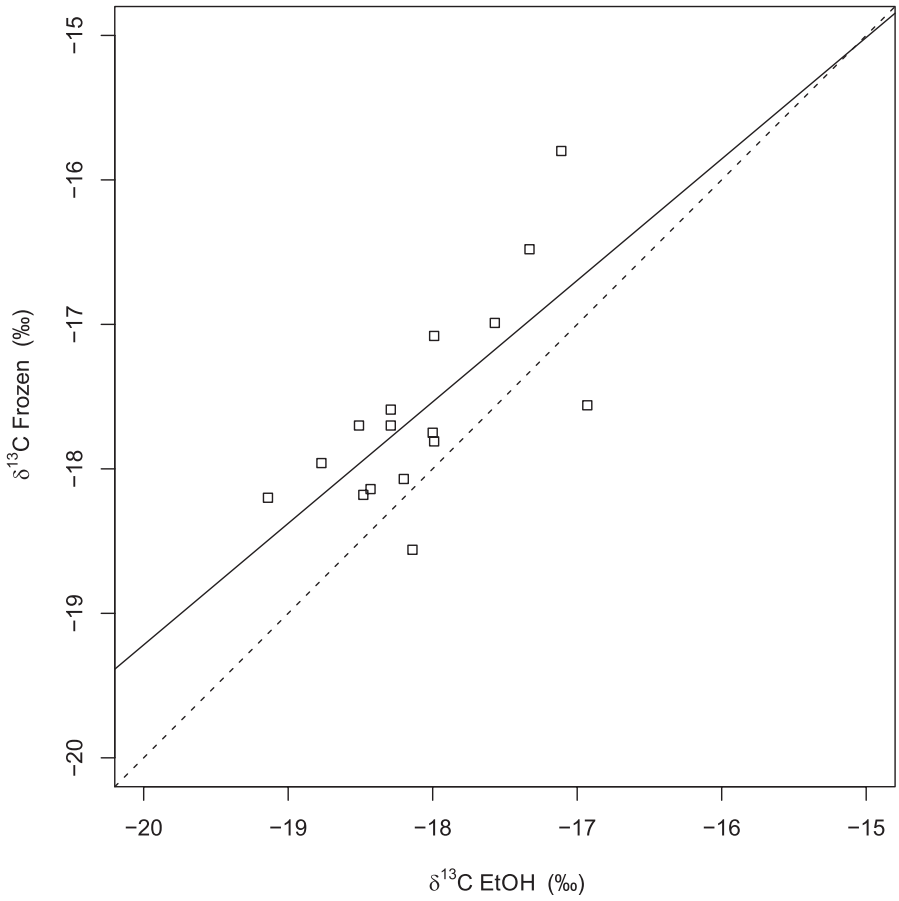

Figure 1. Correlation between $\delta^{13}C$ values from frozen ($\delta^{13}C$ Frozen) and $\delta^{13}C$ ($\delta^{13}C$ EtOH) values from ethanol-preserved common dolphin epidermis samples.

Kelly *et al*. 2006, Bugoni *et al*. 2008, Lesage *et al.* 2010, Ruiz-Cooley *et al*. 2011). Our results are in agreement with some of the previous studies indicating a depleting effect of EtOH on $\delta^{13}C$ values, and a neutral or positive effect on $\delta^{15}N$ values (Hobson *et al.* 1997, Ponsard and Amlou 1999, Kaehler and Pakhomov 2001). Effects were of small magnitude (0.2‰ on average) and were highly predictable for nitrogen isotopes. For carbon, the average depletion (0.5‰) was slightly higher than the analytical error. However, but the spread in the data, particularly at high $\delta^{13}C$ values, suggests that additional data are required to assess the magnitude of effects of EtOH on this isotope, and to confirm that the relationship is linear and thus similar to those described for effects of EtOH on other tissues or species or for other preservatives (see Sarakinos *et al.* 2002, Kelly *et al.* 2006, Barrow *et al.* 2008 for reviews). EtOH preservation is unlikely to hinder the use of nitrogen stable isotopes as an approach to trophic ecology when lipids are extracted prior to analyses; effects of EtOH preservation for carbon isotopes might be more important. As pointed out by

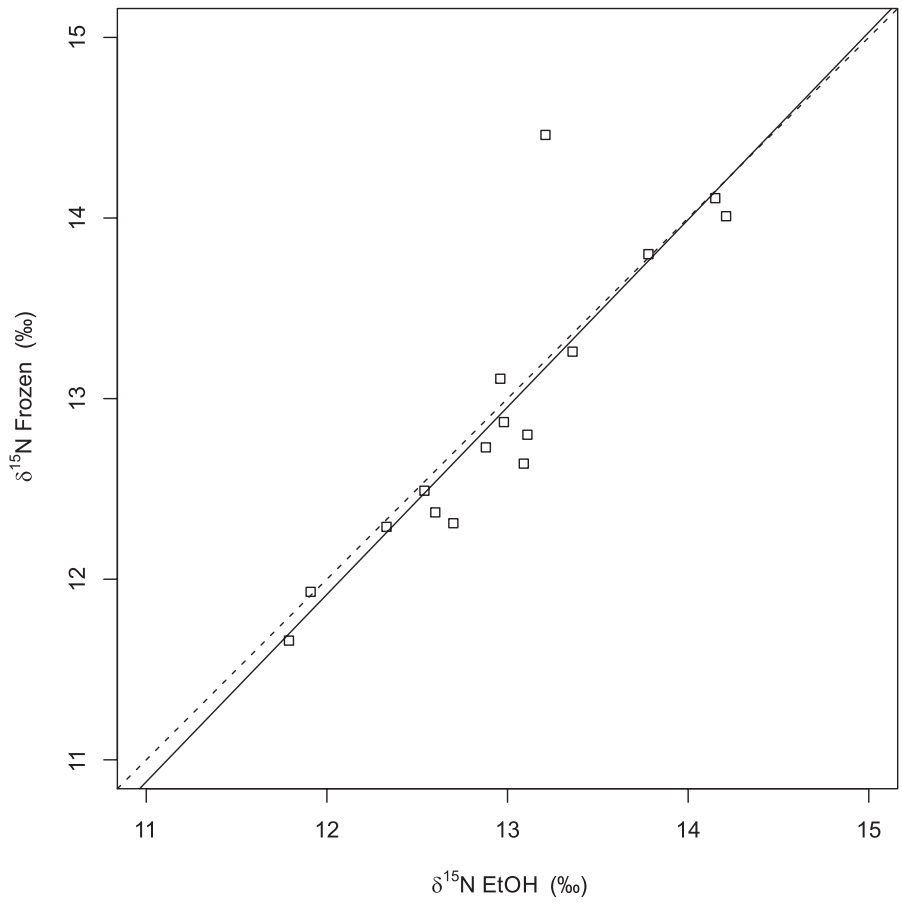

Figure 2. Correlation between δ^{15}N values from frozen (δ^{15}N Frozen) and δ^{15}N (δ^{15}N EtOH) values from ethanol-preserved common dolphin epidermis samples.

several authors (*e.g.,* Lesage *et al*. 2010, Sweeting *et al*. 2004), the significance of biases introduced by a preservative needs to be assessed against the expected magnitude of effects to be documented. Mechanisms behind the observed enrichment or depletion of isotopic signatures are unclear, and may include exchange of light isotopes for heavy ones, extraction of lipids, hydrolysis of proteins during preservation, and contamination by the solvent itself (Carabel *et al.* 2009). Lipid extraction is known to alter both carbon and nitrogen isotope ratios, although to various degrees depending on isotope, tissue, and taxa (Lesage *et al.* 2010). Lipid contents may vary depending on body condition and season; their removal and the use of a tissue with a higher relative proportion of protein allow for a more direct comparison among trophic positions and thus, are generally desirable when studying trophic structure. In counterpart, lipid extraction might have a residual effect on δ^{15}N values and thus, bias trophic structure. In cetaceans, this effect varies between –0.15‰ and +0.30‰ depending on species group (Lesage *et al*. 2010). The lack of a significant difference

in $\delta^{15}N$ values between EtOH-preserved and frozen samples suggests that effects of lipid extraction on $\delta^{15}N$ values in dolphins, a taxonomic group not studied in Lesage *et al*. (2010), is also relatively small, and within the boundary of effects of EtOH, *i.e.*, 0.2‰ on average. An error of this magnitude would introduce a bias of 20% or less in dietary studies relying on isotopic mixing models where sources are separated by 1‰ or more. In other systems, where sources are closer together, biases introduced by solvents or lipid extraction may have a greater impact on dietary results and need to be explicitly accounted for (Lesage *et al*. 2010). The mean residual effect of EtOH for carbon isotopes is equivalent to approximately half a trophic level for marine environments (DeNiro and Epstein 1978, Caut *et al*. 2009), and thus might introduce substantial errors in isotopic mixing models in cases where dietary sources are less than 2‰–3‰ apart (Lesage *et al.* 2010). We conclude that while EtOH preserved samples are unlikely to introduce large errors in trophic studies relying on $\delta^{15}N$ values, this might not be the case for study questions exploiting $\delta^{13}C$ values. This potential source of error needs at least to be taken into account, particularly when using isotopic mixing models to reconstruct dolphin diet.

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