Journal of Mammalogy

Volume 100 | Issue 1 | February 2019

American Society of Mammalogists
www.mammalogy.org

Celebrating 100 Years (1919-2019): A Century of Scientific Discovery

OXFORD
UNIVERSITY PRESS

Online ISSN: 1545-1542
Print ISSN: 0022-2372
jmammal.oxfordjournals.org

Journal of Mammalogy, 100(1):150–157, 2019
DOI:10.1093/jmammal/gyy187
Published online 25 January 2019

Effects of ethanol storage and lipids on stable isotope values in a large mammalian omnivore

Jernej Javornik,* John B. Hopkins III, Saša Zavadlav, Tom Levanič, Sonja Lojen, Tomaž Polak, and Klemen Jerina

Biotechnical Faculty, Department of Forestry, University of Ljubljana, Večna pot 83, 1000 Ljubljana, Slovenia (JJ, JBH, KJ)
School of Biodiversity Conservation, Unity College, 90 Quaker Hill Road, Unity, ME 04988, USA (JBH)
Department of Yield and Silviculture, Slovenian Forestry Institute, Večna pot 2, 1000 Ljubljana, Slovenia (SZ, TL)
Department of Environmental Sciences, Jožef Stefan Institute, Jamova cesta 39, 1000 Ljubljana, Slovenia (SL)
School of Environmental Sciences, University of Nova Gorica, Lanthieri mansion, Glavni trg 8, 5271 Vipava, Slovenia (SL)
Biotechnical Faculty, Department of Food Science and Technology, University of Ljubljana, Jamnikarjeva 101, 1000 Ljubljana, Slovenia (TP)

* *Correspondent: Jernej.Javornik@bf.uni-lj.si*

Ethanol storage and lipid extraction can alter the isotopic composition of animal tissues, which can bias dietary estimates calculated by stable isotope mixing models (SIMMs). We examined the effects of ethanol storage and lipid extraction on δ^{13}C, δ^{15}N, and δ^{34}S values measured in brown bear (*Ursus arctos*) muscles and livers. We also used isotopic data from our experiment to understand the effect of ethanol storage and lipid extraction on dietary contributions calculated by SIMMs. We found that ethanol storage and lipid extraction caused small increases in δ^{13}C values for both muscles (ethanol storage: +0.4 ± 0.5‰, lipid extraction: +0.4 ± 0.4‰) and liver (ethanol storage: +0.6 ± 0.3‰, lipid extraction: +0.8 ± 0.5‰). In contrast, δ^{15}N and δ^{34}S values did not change when stored in ethanol or when lipids were extracted from tissues. Ethanol storage and lipid extraction had negligible effects on estimated dietary contributions. We show that a relatively high lipid content in the muscles and livers of some large-bodied terrestrial omnivores do not necessarily have an effect on dietary estimates that rely on carbon stable isotopes. Our results suggest that ethanol storage could be a valuable alternative method for preserving animal tissue prior to stable isotope analysis when freezing or drying is impractical. Nevertheless, further research is needed on the mechanisms that control changes in stable isotope composition in tissues stored in ethanol. We recommend investigating the effects of ethanol on stable isotope values in species and tissues of interest before storing samples in ethanol.

Key words: lipid extraction, sample preservation, sulfur stable isotopes, *Ursus arctos*

Stable isotope analysis has become a standard method in mammalian ecology (Ben-David and Flaherty 2012a; Walter et al. 2014). Carbon (^{13}C/^{12}C), nitrogen (^{15}N/^{14}N), and sulfur (^{34}S/^{32}S) stable isotope ratios (hereafter expressed as δ^{13}C, δ^{15}N, and δ^{34}S values, respectively) are often used to estimate the dietary contributions of plant- and animal-based foods as well as marine versus terrestrial food sources (Newsome et al. 2007; Ben-David and Flaherty 2012b). The main assumption of such studies is that stable isotope values measured in animal tissues reflect those from their foods during a particular time period (DeNiro and Epstein 1978). Unfortunately, this assumption is not always met, as stable isotopes in animal tissues can be altered by many factors, including storage methods and lipid content (Boecklen et al. 2011).

Ethanol is commonly used as a preservative to store animal tissues (Von Endt 1994). Results from past studies suggest that ethanol storage can affect δ^{13}C and δ^{15}N values in animal tissues, including elasmobranchs, squids (e.g., Ruiz-Cooley et al. 2011; Olin et al. 2014; Stallings et al. 2015), and dolphins (*Delphinus delphis*—Kiszka et al. 2014). In contrast, other studies reported that ethanol storage had no effect on δ^{13}C or δ^{15}N values of muscle, blood, eggs, and epidermis of reptiles, birds, and terrestrial mammals (e.g., Hobson et al. 1997; Gloutney and Hobson 1998; Barrow et al. 2008).

Lipids in animal tissues formed in de novo lipid biosynthesis are depleted in ^{13}C, and consequently, have lower $\delta^{13}C$ values compared to other cell compounds and dietary sources from which they were formed (DeNiro and Epstein 1977). High lipid contents in some tissues may therefore alter the dietary contributions estimated by stable isotope mixing models (SIMMs) (Post et al. 2007). To avoid this unwanted effect, lipids are typically extracted from tissues with chemical extraction prior to conducting stable isotope analyses (Boecklen et al. 2011). Experimental studies that compared lipid-extracted to non-lipid-extracted samples found that the magnitude that lipids affect $\delta^{13}C$ values is positively correlated to the lipid content and carbon to nitrogen ratio of the tissue (hereafter, C:N; e.g., Post et al. 2007; Ruiz-Cooley et al. 2011; Skinner et al. 2016). Unfortunately, lipid extraction is a time-consuming process and can cause unwanted shifts in $\delta^{15}N$ values (Elliott and Elliott 2016). Instead of extracting lipids from tissues, some researchers use C:N as an indicator of lipid content to correct $\delta^{13}C$ values (known as mathematical or lipid normalization; e.g., Kiljunen et al. 2006; Ehrich et al. 2011).

Few studies have focused on understanding the effects of ethanol storage and lipid extraction on $\delta^{13}C$ and $\delta^{15}N$ values of marine (skin and bone: e.g., Lesage et al. 2010; Kiszka et al. 2014; Wilson et al. 2014; Tatsch et al. 2016) and terrestrial (blood, muscles, and livers: domestic sheep [*Ovies aries*], red deer [*Cervus elaphus*], lemmings [*Dicrostonyx* spp.], and artic fox [*Vulpes lagopus*]—Hobson et al. 1997; Post et al. 2007; Ehrich et al. 2011; Yurkowski et al. 2015) mammals. To our knowledge, no studies have investigated the effects of ethanol storage and lipid extraction on $\delta^{34}S$ values. The use of sulfur stable isotopes in the study of mammals is gaining popularity (e.g., Hopkins et al. 2014b, 2017; Mowat et al. 2017), reinforcing the need to understand the impact of storage and lab preparation techniques such as these on $\delta^{34}S$ values.

The purpose of this study was to understand the effect of ethanol storage and lipid extraction on $\delta^{13}C$, $\delta^{15}N$, and $\delta^{34}S$ values of brown bear (*Ursus arctos*) muscle and liver. We concentrated our investigation on muscle and liver because these tissues are commonly collected from large mammals (Boecklen et al. 2011) and are often stored in ethanol. Bears also express high reliance for dietary fats and their metabolism largely depends on storage fats (triglycerides—Erlenbach et al. 2014). We therefore expect to find high triglyceride content (hereafter lipid content) in muscles and livers of bears, leading to significant increases in $\delta^{13}C$ values in these tissues following lipid extraction. We also tested if potential differences in $\delta^{13}C$ values between lipid-extracted and non-lipid-extracted samples can be explained by C:N, as suggested by other studies (e.g., Kiljunen et al. 2006; Ehrich et al. 2011), and demonstrated the effect of ethanol storage and lipid extraction on dietary contributions for brown bears estimated by SIMMs. Lastly, we provide some general guidelines for storing tissues in ethanol and extracting lipids from tissues of mammals, especially omnivores and carnivores that depend on fat in their diets and often have high lipid content in their tissues.

Materials and Methods

Sample collection and preparation.—We used muscle and liver samples from 26 brown bears either harvested by hunters (n = 20) or killed by vehicles (n = 6) in the Dinaric Mountains of southern Slovenia (45°15′–46°15′N, 13°30′–15°15′E) in February–November 2016. Trained personnel of the Slovenian Forestry Service collected ~50 g of both rectus abdominis muscle and liver within a few hours following death. At the time of collection, each sample of muscle and liver was cut in half. One part was stored in a plastic bag and frozen at −20°C. The other part was stored for 5 to 296 days (median: 252 days) at −20°C in a 50-ml self-standing centrifuge tube filled with 30 ml of 96% ethanol-water solution (v/v).

In the lab, we removed any visible adipose tissue, and rinsed all samples with distilled water and freeze-dried them for 24 h. We had two treatment groups and a control group for both muscle and liver (Table 1). We divided frozen muscle and liver samples in two parts: one part was left untreated, representing the control group (hereafter, muscle or liver control), and the other part underwent lipid extraction (hereafter, muscle or liver lipid treatment). Tissues stored in ethanol represented the ethanol treatment (hereafter, muscle or liver ethanol treatment). We extracted lipids using a Soxhlet method (AOAC, 991.36). We weighed freeze-dried samples and transferred them from glass vials into extraction glass microfiber thimbles (649106; Macherey-Nagel SARL, Hoerdt, France) where lipids were removed over the course of 12 h at 97°C using 120 ml of hexane. After extraction, samples were dried overnight. Once the sample weights stabilized, they were transferred into glass vials. We then determined lipid content gravimetrically by calculating changes in sample weight. Lastly, prior to stable isotope analysis, we ground and homogenized all samples using a ball mill (TissueLyserLT; Qiagen, Hilden, Germany).

In fall 2016 and winter 2017, we collected samples of the most important food sources for bears in our study area, as determined by Kavčič et al. (2015; Supplementary Data SD2). Prior to stable isotope analysis, we freeze-dried all samples for 24 h, cut them into smaller pieces using a scalpel, and homogenized all pieces into a fine paste using a mortar and pestle (beech and hazel nuts) or to a fine powder using a ball mill (all other samples).

Table 1.—Treatments applied to brown bear (*Ursus arctos*) muscle (n = 26) and liver (n = 26) samples.

Treatment	Treatment description
Control	Stored at −20°C, rinsed with distilled water, freeze-dried for 24 h
Lipid treatment	Stored at −20°C, rinsed with distilled water, freeze-dried for 24 h, lipid extraction
Ethanol treatment	Stored at −20°C in 30 ml of 96% ethanol-water solution (v/v), rinsed with distilled water, freeze-dried for 24 h

Stable isotope analysis.—We performed stable isotope and elemental concentration analysis using a continuous flow isotope ratio mass spectrometer Isoprime 100 (Isoprime Ltd, United Kingdom, now Elementar GmbH, Hamburg, Germany), coupled with a Vario Pyro Cube elemental analyser (Elementar GmbH). For carbon and nitrogen stable isotope and elemental concentration analysis, we weighed the following into tin capsules: 1.0 ± 0.05 mg of animal tissues, 2.0 ± 0.05 mg of beechnuts, and 3.5 ± 0.05 mg of all other plant material. For sulfur stable isotope analysis of muscle and liver, we weighed 5.0 ± 1.0 mg of tissue into tin capsules. All stable isotopes values are expressed in δ notation as: $\delta^{j/i}X = ({}^{j}X/{}^{i}X)_{sample}/({}^{j}X/{}^{i}X)_{international\ standard} - 1$; where $\delta^{j/i}X$ is $\delta^{13}C$, $\delta^{15}N$, or $\delta^{34}S$ value, ${}^{j}X$ is heavier isotope (^{13}C, ^{15}N, or ^{34}S), and ${}^{i}X$ is lighter isotope (^{12}C, ^{14}N, or ^{32}S). We reported all δ values versus international standards (VPDB for carbon, air for nitrogen, and VCDT for sulfur). We measured a series of certified and in-house reference material to calibrate and control for measured values. For carbon and nitrogen stable isotope analysis, we used USGS-40, USGS-41, and IAEA-N-1 (for nitrogen only) certified reference materials for calibration of measurements. The following in-house reference materials were also used as controls: oak leaves ($\delta^{13}C_{recommended}$ = −28.4‰, $\delta^{13}C_{measured}$ = −28.5 ± 0.1‰; $\delta^{15}N_{recommended}$ = −1.4‰, $\delta^{15}N_{measured}$ = −1.4 ± 0.1‰), spruce needles ($\delta^{13}C_{recommended}$ = −27.1‰, $\delta^{13}C_{measured}$ = −27.2 ± 0.1‰; $\delta^{15}N_{recommended}$ = −4.1‰, $\delta^{15}N_{measured}$ = −4.1 ± 0.1‰), and tobacco leaves ($\delta^{13}C_{recommended}$ = −27.1‰, $\delta^{13}C_{measured}$ = −27.1 ± 0.1‰; $\delta^{15}N_{recommended}$ = +3.8‰, $\delta^{15}N_{measured}$ = +3.8 ± 0.1‰). For the calibration of sulfur stable isotope measurements, we used NBS 127, IAEA SO-5, and IAEA SO-6 certified reference materials. We also used certified (Sercon Ltd, Crewe, United Kingdom) isotope reference materials as controls: sorghum flour ($\delta^{34}S_{recommedned}$ = +10.1‰, $\delta^{34}S_{measured}$ = +9.7 ± 0.8‰), protein ($\delta^{34}S_{recommended}$ = +6.3‰, $\delta^{34}S_{measured}$ = 6.3 ± 0.6‰), and wheat flour ($\delta^{34}S_{recommended}$ = −1.4‰, $\delta^{34}S_{measured}$ = −1.2 ± 0.4‰). Finally, we used acetanilide from Merck KGaA, Germany (% $C_{recommended}$ = 71.1%, % $C_{measured}$ = 71.1 ± 0.4%; % $N_{recommended}$ = 10.4%, % $N_{measured}$ = 10.4 ± 0.1%) and USGS-40 (% $C_{recommended}$ = 40.8%, % $C_{measured}$ = 40.9 ± 0.2%; % $N_{recommended}$ = 9.5%, % $N_{measured}$ = 9.5 ± 0.1%) for the calibration and control of elemental concentration analysis, respectively.

Data preparation and analysis.—We calculated the lipid content of each sample as a percent of its dry mass. We calculated C:N for each sample as the ratio between their measured carbon (% C) and nitrogen (% N) concentrations. We calculated isotopic differences between each treatment (Table 1) and control ($\Delta\delta X_{treatment\text{-}control} = \delta X_{treatment} - \delta X_{control}$) and between lipid and ethanol treatments ($\Delta\delta X_{lipid\text{-}ethanol} = \delta X_{lipid} - \delta X_{ethanol}$). We reported the mean ± *SD* for each group. We then conducted the following four analyses.

We used general linear models to examine the effects of each treatment on stable isotope values and C:N. We considered stable isotope values and C:N as our dependent variables, and treatment, tissue type, an interaction between treatment and tissue type, and bear identity (bear ID) as independent variables. We formulated all possible models (n = 10; Supplementary Data SD3–SD6). We used the Akaike information criterion corrected for small sample size (AICc) for model selection. We considered the best model with the lowest AICc and potentially any other model with ΔAICc score < 2 as informative (Burnham and Anderson 1998, 2002). If treatment was included in the best model, a Tukey's post hoc test was performed on the treatment variable to determine how ethanol and lipid treatment affected stable isotope values or C:N. We visually inspected distribution of residuals for each model to verify the normality and homoscedasticity of the data.

We used a Spearman's correlation test to determine if differences between ethanol treatment and control ($\Delta\delta^{13}C_{ethanol\text{-}control}$) depend on storage time in ethanol.

We used general linear models to investigate the relationship between $\delta^{13}C$ differences between lipid treatment and control ($\Delta\delta^{13}C_{lipid\text{-}control}$ as dependent variable) with both C:N and tissue type as explanatory variables. We removed liver data for two bears from the analysis because they were extreme outliers with C:N of 9.4 and 11.6 (lipid content of 48.9% and 58.7%), and $\Delta\delta^{13}C_{lipid\text{-}control}$ of 1.8‰ and 2.4‰, respectively. We then used the same model selection procedure and visual diagnostics as described in the first analysis.

Lastly, we demonstrated the impact of treatment-caused stable isotope shifts on dietary contributions estimated by SIMMs. We used one-isotope ($\delta^{13}C$), two-endmember models (Supplementary Data SD2) to represent the native- (C_3 foods) and corn-based (C_4 foods) diets of brown bears in Slovenia. These two isotopically distinct food sources are known to be regularly consumed by bears in Slovenia (Kavčič et al. 2013, 2015). This simple one-isotope model could potentially be used in the future to estimate the consumption of corn by brown bears in Slovenia and other parts of Europe. We used discrimination factors from Kurle et al. (2014) to estimate the $\delta^{13}C$ signature of both endmembers (Supplementary Data SD2). We used the R package IsotopeR (Hopkins and Ferguson 2012) to estimate the dietary contributions of each food source. For all models, we used uninformative priors, and ran three MCMC chains with a burn-in of 10^3 draws followed by 10^4 draws from the posterior. We reported the mean, *SD*, and 95% credible interval for each mean marginal posterior density distribution for each food source (i.e., dietary contributions). We then estimated the probability of similarity (POS), defined as the probability that two corn contributions are the same between two treatment groups (i.e., the higher the POS, the greater similarity between diet contributions—Hopkins et al. 2014a).

Results

Treatment effects on stable isotope values.—We found that both treatment and bear ID were included in the best $\delta^{13}C$ model (Table 2; Supplementary Data SD3). We did not consider other models in the candidate set because their ΔAICc scores were > 2 (Supplementary Data SD3). Bear ID explained nearly all the variance associated with $\delta^{13}C$ values (~99%), meaning that differences between bears contributed much more to the observed $\delta^{13}C$ variability than the treatment (Table 2). Results from our post hoc test suggest that $\delta^{13}C$ values increased after ethanol

Table 2.—Analysis of variance (ANOVA) table for the best $\delta^{13}C$ and $\delta^{34}S$ models. We did not consider other models in the candidate set because their ΔAICc scores were > 2 (Supplementary Data SD3–SD5).

Source of variation	*d.f.*	*Sum of squares*	*Mean square*	*F*	*P*
$\delta^{13}C$ model					
Bear ID	25	810.1	32.40	40.906	< 0.01
Treatment	2	11.1	5.54	6.999	< 0.01
Error	128	101.4	0.79		
Total	155				
$\delta^{34}S$ model					
Bear ID	25	28.8	1.2	18.340	< 0.01
Tissue type	1	1.5	1.5	24.664	< 0.01
Treatment	2	0.7	0.3	5.388	< 0.01
Error	127	8.0	0.1		
Total	155				

(P = 0.01) and lipid treatments (P < 0.01) for both muscle ($\Delta\delta^{13}C_{ethanol\text{-}control}$ = +0.4 ± 0.5‰; $\Delta\delta^{13}C_{lipid\text{-}control}$ = +0.4 ± 0.4‰) and liver ($\Delta\delta^{13}C_{ethanol\text{-}control}$ = +0.6 ± 0.3‰; $\Delta\delta^{13}C_{lipid\text{-}control}$ = +0.8 ± 0.5‰). We found no differences between lipid and ethanol treatments (P = 0.87; muscle: $\Delta\delta^{13}C_{lipid\text{-}ethanol}$ = −0.1 ± 0.3‰; liver: $\Delta\delta^{13}C_{lipid\text{-}ethanol}$ = −0.2 ± 0.7‰; Fig. 1; Supplementary Data SD1).

The best $\delta^{15}N$ model (Supplementary Data SD4) included tissue type and bear ID. We did not consider other models in the candidate set because their ΔAICc scores were > 2 (Supplementary Data SD4). This was expected, as both treatments caused negligible shifts in $\delta^{15}N$ values for both muscles ($\Delta\delta^{13}C_{ethanol\text{-}control}$ = +0.1 ± 0.1‰; $\Delta\delta^{13}C_{lipid\text{-}control}$ = 0.0 ± 0.1‰) and livers ($\Delta\delta^{13}C_{ethanol\text{-}control}$ = 0.0 ± 0.2‰; $\Delta\delta^{13}C_{lipid\text{-}control}$ = −0.1 ± 0.2‰; Fig. 1).

We found that treatment, bear ID, and tissue type were included in the best $\delta^{34}S$ model (Table 2; Supplementary Data SD5). We did not consider other models in the candidate set because their ΔAICc scores were > 2 (Supplementary Data SD5). Similar to the $\delta^{13}C$ model, bear ID explained nearly all the model variance (~93%), compared to tissue type (~5%) and treatment (~2%; Table 2). Following lipid extraction, $\delta^{34}S$ values were higher on average (0.2‰) than the control (P = 0.01) and ethanol treatment (P = 0.02), although these shifts are within the analytical precision (≤ 0.8‰) of our reference materials. No difference in $\delta^{34}S$ values were found between ethanol treatment and control (P = 0.97; Fig. 1).

Finally, we found that all factors were included in the best C:N model (Supplementary Data SD6 and SD7). We did not consider other models in the candidate set because their ΔAICc scores were > 2 (Supplementary Data SD6). Liver samples ($C{:}N_{control}$ = 5.3 ± 1.6) had higher C:N compared to muscles ($C{:}N_{control}$ = 3.8 ± 0.4; P < 0.01). The Tukey's post hoc test revealed that both lipid (P < 0.01) and ethanol (P < 0.01) treatments reduced C:N in muscle ($\Delta C{:}N_{lipid\text{-}control}$ = −0.4 ± 0.4; $\Delta C{:}N_{ethanol\text{-}control}$ = −0.2 ± 0.4) and liver ($\Delta C{:}N_{lipid\text{-}control}$ = −0.7 ± 0.3; $\Delta C{:}N_{ethanol\text{-}control}$ = −0.5 ± 0.2).

Effects of storage time in ethanol and lipid normalization of carbon stable isotope values.—Differences in $\delta^{13}C$ values ($\Delta\delta^{13}C_{ethanol\text{-}control}$) did not increase with the duration of ethanol storage for muscle (r_s = 0.22, S = 2277.4, P = 0.28) or liver (r_s = 0.32, S = 1979.7, P = 0.11). The best model for explaining the differences in $\delta^{13}C$ values between lipid treatment and control ($\Delta\delta^{13}C_{lipid\text{-}control}$) included C:N, tissue type, and their interaction. We did not consider other models in the candidate set because their ΔAICc scores were > 2 (Supplementary Data SD8). This result suggests that the relationship between $\Delta\delta^{13}C_{lipid\text{-}control}$ and C:N differs for both tissue types (Supplementary Data SD8). While there was a linear relationship between $\Delta\delta^{13}C_{lipid\text{-}control}$ and C:N for bear muscles, there was no such relationship for bear livers (Fig. 2; Supplementary Data SD9).

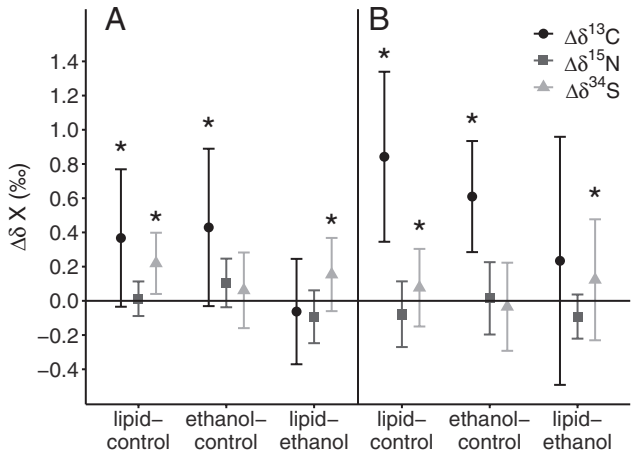

Fig. 1.—Observed differences (mean ± 1 *SD*) in stable isotope values ($\Delta\delta X = \Delta\delta^{13}C$, $\Delta\delta^{15}N$, or $\Delta\delta^{34}S$) between each treatment and control and between treatments for brown bear (*Ursus arctos*) muscles (A) and livers (B). Asterisks denote significant differences.

Effects on estimated dietary contributions.—We found that corn was a major contributor to the diets of brown bears (Table 3). Although we found that estimated mean population-level dietary contributions for bears were slightly higher for both ethanol and lipid treatment groups compared to the control, overall differences in dietary contributions among treatments and controls were small, ranging < 5% of estimated values (Table 3). For livers, we found that the proportions of corn in bear diets were more similar between both treatments (lipid treatment versus ethanol treatment; POS_{liver} = 0.81) than between treatments and the control (ethanol treatment versus control; POS_{liver} = 0.54; lipid treatment versus control; POS_{liver} = 0.56).

In contrast, for muscle, we found that the proportions of corn in bear diets were more similar between both treatments (lipid treatment versus ethanol treatment; POS_{muscle} = 0.81) and between lipid-extracted samples and the control (lipid treatment versus control; POS_{muscle} = 0.82) compared to ethanol treatment and control (ethanol treatment versus control; POS_{muscle} = 0.56).

Discussion

We investigated the isotopic effects of storing brown bear muscle and liver in ethanol and extracting lipids from these tissues. We made three important discoveries. First, we found evidence for small increases of similar magnitude in $\delta^{13}C$ values following both ethanol storage and lipid extraction (Fig. 1); this was not the case for $\delta^{15}N$ or $\delta^{34}S$ values (Fig. 1). Next, we learned that differences in muscle $\delta^{13}C$ values between lipid-extracted and non-lipid-extracted samples were positively correlated to C:N of non-lipid-extracted samples, suggesting that researchers could lipid-corrected $\delta^{13}C$ values in bear muscle based on C:N measurements (Fig. 2). Lastly, $\delta^{13}C$ values increased in tissues following both treatments, but these changes had little effect on estimated dietary contributions (Table 3). Correcting for ethanol storage or lipids prior to SIMM analysis may not be necessary when differences between endmembers are large (e.g., ~15‰), as in our case for brown bears in Slovenia.

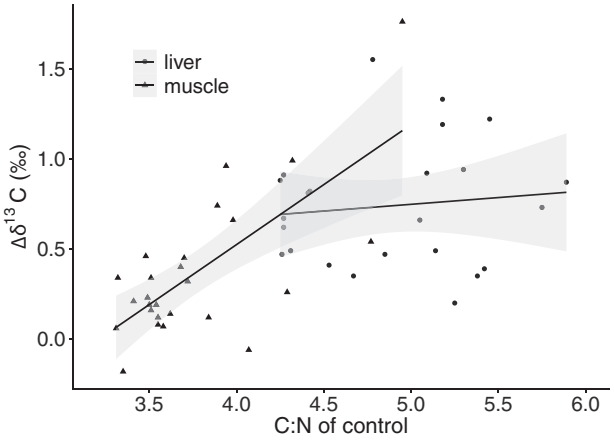

Fig. 2.—Relationships between differences in $\delta^{13}C$ values between lipid extracted and frozen (control) samples ($\Delta\delta^{13}C_{lipid\text{-}control}$) and C:N of control (frozen samples) for brown bear (*Ursus arctos*) muscles (black triangles and line with 95% confidence band) and livers (gray points and line with 95% confidence band). Estimates for model coefficients are presented in Supplementary Data SD9.

Effects of ethanol storage.—We found that ethanol and lipid treatments caused similar increases in $\delta^{13}C$ values for both muscle and liver (Fig. 1), and C:N decreased in these tissues following ethanol storage. Increases in $\delta^{13}C$ values and decreases in C:N following ethanol storage have also been reported in other studies, which suggests that such changes are likely due to ethanol extraction of ^{13}C-depleted, carbon-rich (contributing to high C:N) lipids from tissues (Kaehler and Pakhomov 2001; Sweeting et al. 2004). A study that analyzed the organic compounds leaching from mammal tissues stored in ethanol found a variety of lipids and their constituent fatty acids in the ethanol storage media, showing that some polar lipids, such as triglycerides, are extracted by ethanol (Von Endt 1994). Although we did not extract lipids from ethanol-stored samples, which could have provided stronger evidence that ethanol actually extracts lipids, we suspect, based on our results and those from past studies, that at least some lipids were extracted from samples stored in ethanol (Kaehler and Pakhomov 2001; Sweeting et al. 2004). Further research is required to understand the mechanisms behind ethanol-induced changes in $\delta^{13}C$ values. For instance, highly (96%) concentrated ethanol used in our study could also contribute to observed changes, and results could be different using a less-concentrated ethanol (Ponsard and Amlou 1999; Sweeting et al. 2004). We recommend a study that examines the effects of different concentrations of ethanol on the isotope values of numerous animal tissues.

Unlike fish and squid muscle (Sweeting et al. 2004; Ruiz-Cooley et al. 2011; Olin et al. 2014; Stallings et al. 2015), ethanol storage did not affect $\delta^{15}N$ values of brown bear muscle and liver in this study or tissues sampled from other mammals, birds, and reptiles (Hobson et al. 1997; Gloutney and Hobson 1998; Barrow et al. 2008). We also found that the isotopic effect of ethanol storage was within the measurement error limits for $\delta^{34}S$ values, indicating that storing mammal tissues in ethanol prior to stable isotope analysis did not alter their $\delta^{34}S$ values.

Similar to past studies, we did not find a correlation between increases in $\delta^{13}C$ values and storage time in ethanol (Kaehler and Pakhomov 2001; Sarakinos et al. 2002). Sweeting et al. (2004) and Kiszka et al. (2014) reported that shifts in $\delta^{13}C$

Table 3.—Proportional dietary contributions (%) for brown bears (*Ursus arctos*) harvested or killed in Slovenia in February–November 2016. Analyses were conducted using liver or muscle tissue, in three treatments prior to analysis (control, ethanol storage, lipid extraction; see Table 1 and text for descriptions).

	Liver				Muscle			
	Mean	1 *SD*	2.5%	97.5%	Mean	1 *SD*	2.5%	97.5%
Control								
Corn	25.3	4.5	16.2	34.3	25.7	3.2	19.5	31.9
C_3 foods	74.7	4.5	65.7	83.8	74.3	3.2	68.1	80.5
Ethanol treatment								
Corn	29.5	5.2	16.5	38.3	28.8	3.0	23.0	34.4
C_3 foods	70.5	5.2	61.7	83.5	71.2	3.0	65.6	77.0
Lipid treatment								
Corn	31.9	4.4	23.2	40.7	28.1	3.2	21.8	34.0
C_3 foods	68.1	4.4	59.3	76.8	71.9	3.2	66.0	78.2

values for fish tissues and dolphin epidermis following ethanol storage were independent of storage duration, and increases in $\delta^{13}C$ values of fish, octopi, and freshwater clams occurred within days when they were stored in ethanol (Kaehler and Pakhomov 2001; Sarakinos et al. 2002).

Effects of lipid extraction.—Some studies suggest that increases in $\delta^{13}C$ values are positively correlated to lipid content in tissues (e.g., Post et al. 2007). We observed smaller increases in $\delta^{13}C$ values following lipid extraction than expected, according to the high lipid content measured in both muscles (mean ± *SD*; 11.9 ± 5.5%) and livers (17.9 ± 11.5%—Post et al. 2007). It is therefore likely that not only lipid content, but also species-specific factors, including metabolic pathways, could influence changes in $\delta^{13}C$ values following lipid extraction. For instance, Rode et al. (2016) found evidence that some carbon from lipids is routed for biosynthesis of nonessential amino acids in brown bears. In our study, small difference in $\delta^{13}C$ values following lipid extraction also could be attributed to carbon from lipids being routed to the biosynthesis of certain amino acids.

We found that our lipid extraction method did not have an effect on $\delta^{15}N$ and $\delta^{34}S$ values of brown bear muscle and liver tissues. The reported effects of lipid extraction on $\delta^{15}N$ values in the literature vary significantly in magnitude and direction, and these discrepancies can likely be attributed to the method used for lipid extraction (Boecklen et al. 2011). A number of methods have been used to extract lipids from tissues prior to stable isotope analysis, but there is currently no consensus to standardize this lab procedure (see Wilson et al. 2014 for review). Unlike hexane and other lipid extraction solvents (e.g., Bodin et al. 2007), polar solvents, such as chloroform-methanol, have been found to increase $\delta^{15}N$ values in tissues (e.g., Yurkowski et al. 2015; Gimenez et al. 2017). Strong polar solvents extract a substantial amount of non-lipids (≤ 35%—Dobush et al. 1985), including some hydrophobic essential amino acids, which are not enriched in ^{15}N with each trophic level (Elliott and Elliott 2016). However, such solvents are commonly used for lipid extraction as they extract both nonpolar lipids (e.g., triglycerides) and polar lipid compounds (e.g., phospholipids and free fatty acids—Schlechtriem et al. 2003; Doucette et al. 2010). It is likely that no change in $\delta^{15}N$ (and possibly $\delta^{34}S$) values following lipid extraction could be attributed to the method we used, and that other lipid extraction methods could yield different results.

C:N has generally been described as an indicator of lipid content in animal tissues (Post et al. 2007), but growing evidence, including results from this study, suggests otherwise. Similar to other experimental studies of mammals, we found that there is a linear relationship between the difference in $\delta^{13}C$ values following lipid extraction and C:N for muscles; however, we did not find such relationship for liver (Ehrich et al. 2011; Yurkowski et al. 2015; Fig. 2). It is incorrect to assume that lipid content in the tissue is the only factor contributing to the variability of C:N. For example, glycogen can contribute to high elemental concentrations of carbon in tissues, such as liver, leading to high C:N (Kiljunen et al. 2006; Patterson and Carmichael 2016). In summary, our results indicate that C:N is not a good indicator of lipid content in brown bear livers, especially when lipid content is ≤ 25%.

We found that the liver lipid contents of two large, adult males were very high (48.9% and 58.7%). Measurement error may not be responsible for these two outliers because of correspondingly high liver C:N (9.4 and 11.6), differences in $\delta^{13}C$ value following lipid extraction ($\Delta\delta^{13}C_{\text{lipid-control}}$: 1.8‰ and 2.4‰), and a much lower water content in these samples (54.4% and 48.9%; mean ± *SD* water content for all other samples: 67.4 ± 2.4%). To our knowledge, this is the highest liver lipid content measured in a bear (*Ursus* spp.—Gebbink et al. 2008). We suspect that high lipid content in livers could be attributed to the consumption of energy-rich foods that are high in fat and carbohydrates. Further research to determine what causes such high lipid content in bear livers would be interesting, as such lipid content has a strong effect on their $\delta^{13}C$ values ($\Delta\delta^{13}C_{\text{lipid-control}}$ ~ 2‰). Nevertheless, such samples can be detected due to their high C:N (> 9; either frozen or ethanol-stored samples) and can be either excluded or lipid-extracted and measured again.

Ethanol storage and lipid extraction had negligible effect on estimated dietary parameters. Both ethanol storage and lipid extraction yielded similar dietary contributions (Table 3) because differences in $\delta^{13}C$ values were small between both treatments (Fig. 1). The smaller the difference in stable isotope values between treatments relative to the differences in stable isotope values between endmembers, the smaller the error introduced to estimated dietary contributions (Lesage et al. 2010; Giménez et al. 2017). In our study, the difference in $\delta^{13}C$ values between endmembers was large (~15‰; Supplementary Data SD2) compared to the difference in $\delta^{13}C$ values between treatments (Fig. 1). It is therefore expected that the differences between treatment dietary contributions were small (~3% for muscle ethanol and lipid treatment and ~6% for liver ethanol and lipid treatment; Table 3; see Post et al. 2007), suggesting that ethanol storage and lipid content had negligible effects on population-level dietary contributions estimated by one-isotope ($\delta^{13}C$) two-source (C_3 and C_4) SIMMs.

Guidelines for storing and extracting lipids from mammal tissues.—Ethanol storage is a valuable storage technique, especially when freezing or drying tissue samples is impractical (e.g., working in remote areas or when samples are collected and stored by volunteers, such as hunters—Ponsard and Amlou 1999; Skrbinšek et al. 2012) or when samples need to be archived (Sarakinos et al. 2002). Ethanol does not affect $\delta^{15}N$ (Hobson et al. 1997; Kiszka et al. 2014; this study) or $\delta^{34}S$ (this study) values in mammal tissues. However, it likely extracts lipids from those tissues, which can increase sample $\delta^{13}C$ values, but this mechanism is not fully understood (e.g., Kaehler and Pakhomov 2001; Sweeting et al. 2004; Ruiz-Cooley et al. 2011; this study). Therefore, we suggest conducting a similar study as presented here to verify the extent in which ethanol affects $\delta^{13}C$ values in tissues of specific species of interest before storing samples in ethanol. We also recommend that researchers avoid using highly concentrated ethanol for tissue storage when lipid extraction is unwanted

prior to stable isotope analysis, as ethanol may extract lipids from stored samples (Von Endt 1994).

Lipids are an important dietary macronutrient for carnivorous and omnivorous mammals; they are used not only for energy metabolism but also for biosynthesis of tissues (Newsome et al. 2014; Rode et al. 2016). Prior to conducting stable isotope analyses, we suggest researchers carefully consider if lipid extraction or mathematical normalization is necessary. Although we found little change to estimated dietary parameters generated by SIMMs following lipid extraction, differences could have been significant if δ^{13}C values between endmembers were smaller (< 10‰—Post et al. 2007; Lesage et al. 2010; Giménez et al. 2017).

Acknowledgments

This study was financed by the Slovenian Research Agency (project no. J4-7362, a Ph.D. scholarship to JJ, and two other research programs: P4-0107 [TL and SZ] and P1-0143 [SL]). We are grateful to employees of the Slovenian Forestry Service for diligently collecting and handling tissue samples. We also acknowledge the Associate Editor, R. Constantine, and two anonymous reviewers for providing useful comments and edits. Lastly, we want to thank D. Bordjan, M. Burnik-Šturm, M. Di Bitteti, U. Fležar, L. Gál, M. Krofel, E. Polaina, M. Rodriguez Recio, N. Škrk, L. Vranković, and A. Žagar for help in the lab and for providing comments associated with this manuscript.

Supplementary Data

Supplementary data are available at *Journal of Mammalogy* online.

Supplementary Data SD1.—Stable isotope data derived from bear (*Ursus arctos*) muscle and liver for each treatment and control.

Supplementary Data SD2.—Data used in stable isotope mixing model (SIMM) analysis.

Supplementary Data SD3.—Model selection results for δ^{13}C general linear models.

Supplementary Data SD4.—Model selection results for δ^{15}N general linear models.

Supplementary Data SD5.—Model selection results for δ^{34}S general linear models.

Supplementary Data SD6.—Model selection results for C:N general linear models.

Supplementary Data SD7.—Analysis of variance (ANOVA) table for the best C:N model.

Supplementary Data SD8.—Model selection results for general linear models used to investigate the relationship between $\Delta\delta^{13}C_{lipid\text{-}control}$, C:N, and tissue type.

Supplementary Data SD9.— Estimated coefficients and intercepts for explanatory variables included in the best general linear model that explains the isotopic (δ^{13}C) differences between lipid treatment and control in brown bear (*Ursus arctos*) muscles and liver.

Literature Cited

Barrow, L. M., K. A. Bjorndal, and K. J. Reich. 2008. Effects of preservation method on stable carbon and nitrogen isotope values. Physiological and Biochemical Zoology 81:688–693.

Ben-David, M., and E. A. Flaherty. 2012a. Theoretical and analytical advances in mammalian isotope ecology: an introduction: fig. 1. Journal of Mammalogy 93:309–311.

Ben-David, M., and E. A. Flaherty. 2012b. Stable isotopes in mammalian research: a beginner's guide. Journal of Mammalogy 93:312–328.

Bodin, N., F. Le Loc'h, and C. Hily. 2007. Effect of lipid removal on carbon and nitrogen stable isotope ratios in crustacean tissues. Journal of Experimental Marine Biology and Ecology 341:168–175.

Boecklen, W. J., C. T. Yarnes, B. A. Cook, and A. C. James. 2011. On the use of stable isotopes in trophic ecology. Annual Review of Ecology, Evolution, and Systematics 42:411–440.

Burnham, K. P., and D. R. Anderson. 1998. Model selection and inference. 1st ed. Springer-Verlag, New York.

Burnham, K. P., and D. R. Anderson. 2002. Model selection and multimodel inference: a practical information-theoretic approach. 2nd ed. Springer-Verlag, New York.

DeNiro, M. J., and S. Epstein. 1977. Mechanism of carbon isotope fractionation associated with lipid synthesis. Science 197:261–263.

DeNiro, M. J., and S. Epstein. 1978. Influence of diet on the distribution of carbon isotopes in animals. Geochimica et Cosmochimica Acta 42:495–506.

Dobush, G. R., C. D. Ankney, and D. G. Krementz. 1985. The effect of apparatus, extraction time, and solvent type on lipid extractions of snow geese. Canadian Journal of Zoology 63:1917–1920.

Doucette, J. L., B. Wissel, and C. M. Somers. 2010. Effects of lipid extraction and lipid normalization on stable carbon and nitrogen isotope ratios in double-crested cormorants: implications for food web studies. Waterbirds 33:273–284.

Ehrich, D., et al. 2011. Stable isotope analysis: modelling lipid normalization for muscle and eggs from arctic mammals and birds. Methods in Ecology and Evolution 2:66–76.

Elliott, K. H., and J. E. Elliott. 2016. Lipid extraction techniques for stable isotope analysis of bird eggs: chloroform-methanol leads to more enriched 13C values than extraction via petroleum ether. Journal of Experimental Marine Biology and Ecology 474:54–57.

Erlenbach, J. A., K. D. Rode, D. Raubenheimer, and C. T. Robbins. 2014. Macronutrient optimization and energy maximization determine diets of brown bears. Journal of Mammalogy 95: 160–168.

Gebbink, W. A., et al. 2008. Tissue-specific congener composition of organohalogen and metabolite contaminants in East Greenland polar bears (*Ursus maritimus*). Environmental Pollution 152:621–629.

Giménez, J., F. Ramírez, M. G. Forero, J. Almunia, R. de Stephanis, and J. Navarro. 2017. Lipid effects on isotopic values in bottlenose dolphins (*Tursiops truncatus*) and their prey with implications for diet assessment. Marine Biology 164:1–9.

Gloutney, M. L., and K. A. Hobson. 1998. Field preservation techniques for the analysis of stable-carbon and nitrogen isotope ratios in eggs. Journal of Field Ornithology 69:223–227.

Hobson, K. A., M. L. Gloutney, and H. L. Gibbs. 1997. Preservation of blood and tissue samples for stable-carbon and stable-nitrogen isotope analysis. Canadian Journal of Zoology 75:1720–1723.

Hopkins, J. B., and J. M. Ferguson. 2012. Estimating the diets of animals using stable isotopes and a comprehensive Bayesian mixing model. PLoS One 7:e28478.

Hopkins, J. B., J. M. Ferguson, D. B. Tyers, and C. M. Kurle. 2017. Selecting the best stable isotope mixing model to estimate grizzly bear diets in the Greater Yellowstone Ecosystem. PLoS One 12:1–19.

Hopkins, J. B., P. L. Koch, J. M. Ferguson, and S. T. Kalinowski. 2014a. The changing anthropogenic diet of American black bear over the past century in Yosemite National Park. Frontiers in Ecology and the Environment 12:107–114.

Hopkins, J. B., J. Whittington, A. P. Clevenger, M. A. Sawaya, and C. C. St. Clair. 2014b. Stable isotopes reveal rail-associated behavior in a threatened carnivore. Isotopes in Environmental and Health Studies 50:322–331.

Kaehler, S., and E. A. Pakhomov. 2001. Effects of storage and preservation on the $\delta^{13}C$ and $\delta^{15}N$ signatures of selected marine organisms. Marine Ecology Progress Series 219:299–304.

Kavčič, I., M. Adamič, P. Kaczensky, M. Krofel, and K. Jerina. 2013. Supplemental feeding with carrion is not reducing brown bear depredations on sheep in Slovenia. Ursus 24:111–119.

Kavčič, I., M. Adamič, P. Kaczensky, M. Krofel, M. Kobal, and K. Jerina. 2015. Fast food bears: brown bear diet in a human-dominated landscape with intensive supplemental feeding. Wildlife Biology 21:1–8.

Kiljunen, M., J. Grey, T. Sinisalo, C. Harrod, H. Immonen, and R. I. Jones. 2006. A revised model for lipid-normalizing $\delta^{13}C$ values from aquatic organisms, with implications for isotope mixing models. Journal of Applied Ecology 43:1213–1222.

Kiszka, J., V. Lesage, and V. Ridoux. 2014. Effect of ethanol preservation on stable carbon and nitrogen isotope values in cetacean epidermis: implication for using archived biopsy samples. Marine Mammal Science 30:788–795.

Kurle, C. M., P. L. Koch, B. R. Tershy, and D. A. Croll. 2014. The effects of sex, tissue type, and dietary components on stable isotope discrimination factors ($\Delta^{13}C$ and $\Delta^{15}N$) in mammalian omnivores. Isotopes in Environmental and Health Studies 50:307–321.

Lesage, V., Y. Morin, È. Rioux, C. Pomerleau, S. H. Ferguson, and É. Pelletier. 2010. Stable isotopes and trace elements as indicators of diet and habitat use in cetaceans: predicting errors related to preservation, lipid extraction, and lipid normalization. Marine Ecology Progress Series 419:249–265.

Mowat, G., P. J. Curtis, and D. J. Lafferty. 2017. The influence of sulfur and hair growth on stable isotope diet estimates for grizzly bears. PLoS One 12:e0172194.

Newsome, S. D., C. Martinez del Rio, S. Bearhop, and D. L. Phillips. 2007. A niche for isotope ecology. Frontiers in Ecology and the Environment 5:429–436.

Newsome, S. D., N. Wolf, J. Peters, and M. L. Fogel. 2014. Amino acid δ13C analysis shows flexibility in the routing of dietary protein and lipids to the tissue of an omnivore. Integrative and Comparative Biology 54:890–902.

Olin, J. A., G. R. Poulakis, P. W. Stevens, J. A. DeAngelo, and A. T. Fisk. 2014. Preservation effects on stable isotope values of archived elasmobranch fin tissue: comparisons between frozen and ethanol-stored samples. Transactions of the American Fisheries Society 143:1569–1576.

Patterson, H. K., and R. H. Carmichael. 2016. The effect of lipid extraction on carbon and nitrogen stable isotope ratios in oyster tissues: implications for glycogen-rich species. Rapid Communications in Mass Spectrometry 30:2594–2600.

Ponsard, S., and M. Amlou. 1999. Effects of several preservation methods on the isotopic content of *Drosophila* samples. Comptes Rendus de l'Academie des Sciences - Serie III 322:35–41.

Post, D. M., C. A. Layman, D. A. Arrington, G. Takimoto, J. Quattrochi, and C. G. Montaña. 2007. Getting to the fat of the matter: models, methods and assumptions for dealing with lipids in stable isotope analyses. Oecologia 152:179–189.

Rode, K. D., et al. 2016. Isotopic incorporation and the effects of fasting and dietary lipid content on isotopic discrimination in large carnivorous mammals. Physiological and Biochemical Zoology 89:182–197.

Ruiz-Cooley, R. I., K. Y. Garcia, and E. D. Hetherington. 2011. Effects of lipid removal and preservatives on carbon and nitrogen stable isotope ratios of squid tissues: implications for ecological studies. Journal of Experimental Marine Biology and Ecology 407:101–107.

Sarakinos, H. C., M. L. Johnson, and M. J. Vander Zanden. 2002. A synthesis of tissue-preservation effects on carbon and nitrogen stable isotope signatures. Canadian Journal of Zoology 80:381–387.

Schlechtriem, C. H., U. Focken, and K. Becker. 2003. Effect of different lipid extraction methods on $\delta^{13}C$ of lipid and lipid-free fractions of fish and different fish feeds. Isotopes in Environmental and Health Studies 39:135–140.

Skinner, M. M., A. A. Martin, and B. C. Moore. 2016. Is lipid correction necessary in the stable isotope analysis of fish tissues? Rapid Communications in Mass Spectrometry 30:881–889.

Skrbinšek, T., M. Jelenčič, L. Waits, I. Kos, K. Jerina, and P. Trontelj. 2012. Monitoring the effective population size of a brown bear (*Ursus arctos*) population using new single-sample approaches. Molecular Ecology 21:862–875.

Stallings, C. D., et al. 2015. Effects of preservation methods of muscle tissue from upper-trophic level reef fishes on stable isotope values ($\delta^{13}C$ and $\delta^{15}N$). PeerJ 3:e874.

Sweeting, C. J., N. V. Polunin, and S. Jennings. 2004. Tissue and fixative dependent shifts of $\delta^{13}C$ and $\delta^{15}N$ in preserved ecological material. Rapid Communications in Mass Spectrometry 18:2587–2592.

Tatsch, A. C., E. R. Secchi, and S. Botta. 2016. Effects of acidification, lipid removal and mathematical normalization on carbon and nitrogen stable isotope compositions in beaked whale (*Ziphiidae*) bone. Rapid Communications in Mass Spectrometry 30:460–466.

Von Endt, D. W. 1994. Spirit collections: a preliminary analysis of some organic materials found in the storage fluids of mammals. Collection Forum 10:10–19.

Walter, W. D., C. M. Kurle, and J. B. Hopkins. 2014. Applications of stable isotope analysis in mammalian ecology. Isotopes in Environmental and Health Studies 50:287–290.

Wilson, R. M., J. P. Chanton, B. C. Balmer, and D. P. Nowacek. 2014. An evaluation of lipid extraction techniques for interpretation of carbon and nitrogen isotope values in bottlenose dolphin (*Tursiops truncatus*) skin tissue. Marine Mammal Science 30:85–103.

Yurkowski, D. J., N. E. Hussey, C. Semeniuk, S. H. Ferguson, and A. T. Fisk. 2015. Effects of lipid extraction and the utility of lipid normalization models on $\delta^{13}C$ and $\delta^{15}N$ values in Arctic marine mammal tissues. Polar Biology 38:131–143.

Submitted 14 August 2018. Accepted 24 December 2018.

Associate Editor was Rochelle Constantine.