Research Article

Rapid Communications in Mass Spectrometry

Received: 3 August 2016 Revised: 26 September 2016 Accepted: 1 December 2016 Published online in Wiley Online Library

Rapid Commun. Mass Spectrom. **2017**, *31*, 389–395
(wileyonlinelibrary.com) DOI: 10.1002/rcm.7802

Effects of decomposition on carbon and nitrogen stable isotope values of muscle tissue of varying lipid content from three aquatic vertebrate species

David J. Yurkowski*,†, Anna J. Hussey, Nigel E. Hussey and Aaron T. Fisk

Great Lakes Institute for Environmental Research, University of Windsor, Windsor, Ontario N9B 3P4, Canada

RATIONALE: Stable isotopes are a prominent tool in animal ecology where data is obtained from analyzing animal tissues, which are typically stored prior to analysis. However, the effect of decomposition on the reliability of stable isotope ratios from animal tissue prior to storage has been seldom studied. Here, we examine the long-term effects of freezing and decomposition of animal tissue on $\delta^{13}C$ and $\delta^{15}N$ values across three different aquatic species of varying lipid content.
METHODS: Ringed seal, lake trout and Greenland shark muscle were divided into different treatment groups and analyzed for their $\delta^{13}C$ values, carbon content (%C), $\delta^{15}N$ values, and nitrogen content (%N) at specific time intervals. The intervals included days 0, 128 and 700 for the frozen storage treatment and at days 0, 1, 2, 4, 8, 16, 32, 64, 128 and 256 for the tissue decomposition treatment in open and closed vials at room temperature.
RESULTS: The difference in $\delta^{13}C$ and $\delta^{15}N$ values between the control and days 128 and 700 for the frozen treatment was minimal and not significant for any species. Generally, significant decreases in carbon (%C) and nitrogen (%N) content and significant increases (>0.5‰) in $\delta^{13}C$ and $\delta^{15}N$ values occurred for muscle of each species left to decompose for 256 days, probably due to the preferential uptake of lighter isotopes during decomposition by microbes. However, the magnitude of change in the $\delta^{13}C$ and $\delta^{15}N$ values up to 8 days in both treatments was low (generally ≤0.1‰) and not significant across most species.
CONCLUSIONS: Freezing for extended time periods (up to 700 days) is a viable storage technique for stable isotope analysis of aquatic animal muscle tissue across a range of lipid contents. Muscle tissue left to decompose at room temperature showed no significant change in $\delta^{13}C$ and $\delta^{15}N$ values after 8 days, and such tissues would still be reliable for ecological interpretations. However, caution should be used for decomposed tissue for >8 days as the $\delta^{13}C$ and $\delta^{15}N$ values will probably be artificially high. Copyright © 2016 John Wiley & Sons, Ltd.

Stable isotopes have become a prominent tool in ecological studies to answer a wide variety of questions encompassing interactions between animals and their environment due to the acquisition and elimination of natural elements through biochemical processes.[1,2] Stable carbon ($\delta^{13}C$ values) and nitrogen ($\delta^{15}N$ values) isotope ratios are the most commonly used isotopes by ecologists. Using $\delta^{13}C$ values, primary productivity can be traced between terrestrial and marine carbon sources, and within aquatic systems, to discriminate between different habitats, such as inshore/offshore and benthic/pelagic.[3,4] Consumer diet and trophic position can be quantified using $\delta^{15}N$ values as protein in consumer tissues has a higher $\delta^{15}N$ value than dietary protein due to the preferential excretion of ^{14}N via nitrogen metabolism.[5]

Isotopic data can be obtained from animal tissue, allowing retrospective analysis of archived tissues. Sample preservation techniques include freezing, freeze- or oven-drying, or storage in chemicals, the latter of which can alter the isotopic composition of the sample.[6–8] Freezing or freeze- and oven-drying are the preferred preservation techniques of animal tissue with no previous effects on stable isotope ratios having been reported.[6,8–10] However, the effects of tissue decomposition on $\delta^{13}C$ and $\delta^{15}N$ values have seldom been studied in stable isotope ecology[11,12] despite the possibility of tissue decomposition prior to storage, particularly in field collections. For example, in logistically difficult study areas, such as the Arctic, it can be challenging to acquire and properly store animal tissues without degradation and the use of solvents. Animal tissue could also degrade after proper storage as a result of laboratory freezer breakdowns. Due to the paucity of data examining the effects of animal tissue decomposition on $\delta^{13}C$ and $\delta^{15}N$ values, we examine this phenomenon for three different aquatic species.

The objective of this study was to investigate the reliability of $\delta^{13}C$ and $\delta^{15}N$ values of animal tissues after long-term storage in freezers as well as decomposition at room temperature over time. Specifically, we quantify the effects of long-term storage in freezers and decomposition on the $\delta^{13}C$ and $\delta^{15}N$ values of muscle tissue from three aquatic vertebrate species, ringed seals (*Pusa hispida*), lake trout (*Salvelinus namaycush*) and Greenland sharks (*Somniosus microcephalus*), over 700 and 256 days, respectively. We predict

* *Correspondence to:* D. J. Yurkowski, University of Manitoba, Department of Biological Sciences, 50 Sifton Rd, Winnipeg, MB R3T 2N2, Canada.
E-mail: dyurkowski1@gmail.com

† Current address: University of Manitoba, Department of Biological Sciences, 50 Sifton Rd, Winnipeg, MB R3T 2N2, Canada.

that muscle tissue preservation through long-term storage in freezers will not affect $\delta^{13}C$ and $\delta^{15}N$ values for each species, whereas muscle decomposition at room temperature will increase the $\delta^{13}C$ and $\delta^{15}N$ values for each species over time due to the preferential uptake of lighter isotopes by microbes. Muscle lipid content differs between these species – it is highest in Greenland sharks[13] compared with lake trout and ringed seals[14,15] – allowing an assessment of whether varying lipid contents can affect the stable isotopic integrity of decomposing tissue. Since lipids provide an oxygen or moisture barrier in muscle,[16] we predict that changes in $\delta^{13}C$ and $\delta^{15}N$ values over time will be higher in ringed seals and lake trout than in Greenland sharks.

EXPERIMENTAL

Sample collection

One ringed seal was collected from an Inuit subsistence hunt and one Greenland shark from a longline set from a commercial fishing vessel in August 2010 near Pangnirtung, Nunavut, Canada (66° 8′ 52″ N, 65° 41′ 58″ W). One lake trout was collected from seine netting in Lake Ontario, Canada (43° 42′ 0″ N, 77° 54′ 0″ W). All muscle samples from each species were immediately stored on ice in the field and then stored frozen at –20°C within hours of capture and sampling.

Treatment preparation

Muscle tissue weighing approximately 20 grams wet weight from each species was subsampled into 67 samples and then divided into three different treatment groupings to determine the effects of storage through freezing and tissue decomposition at room temperature (~21°C) over time on $\delta^{13}C$ and $\delta^{15}N$ values. The first set (n = 3) was the control group (i.e. day 0) for each species and these samples were freeze-dried when frozen, homogenized and then analyzed before lipid extraction (i.e. BULK). We then lipid-extracted these BULK control samples for another control (lipid-extracted) using 2 mL of 2:1 chloroform/methanol solvent similar to the Bligh and Dyer[17] method and detailed in McMeans *et al.*[18] The second set (n = 10 per species) was placed into sterile cryovials and then stored at –20°C with a lid on the vial. Five frozen samples were taken at day 128 and then at day 700 for freeze-drying and homogenization with a mortar and pestle prior to stable isotope analysis. These tissue samples were not lipid-extracted prior to analysis and their $\delta^{13}C$ and $\delta^{15}N$ values were compared with those of BULK samples from the control group (i.e. day 0). The third set (n = 54 samples per species) was placed into sterile cryovials and stored at room temperature (~21°C) within a fume-hood. These samples were then subdivided into an open vial (n = 27 per species) and closed vial (n = 27 per species) group over the course of the study to test for differences with aridity. The open vial treatment mimicked a slow drying process while degrading, whereas the closed vial treatment represented a moist environment. At intervals of 1, 2, 4, 8, 16, 32, 64, 128 and 256 days, 3 vials were selected from each group for freeze-drying, homogenization, lipid extraction and stable isotope analysis. The $\delta^{13}C$ and $\delta^{15}N$ values of these samples were then compared with those of lipid-extracted samples from the control group (i.e. day 0).

Stable isotope analysis

After tissue processing for each sample, 400–600 µg of muscle tissue was weighed into tin capsules for stable isotope analysis. The $\delta^{13}C$ and $\delta^{15}N$ values were measured using a ThermoFinnigan Delta^{Plus} mass spectrometer (ThermoFinnigan, San Jose, CA, USA) coupled with an elemental analyzer (Costech, Valencia, CA, USA) at the Chemical Tracers Laboratory, Great Lakes Institute for Environmental Research, University of Windsor (Windsor, Canada). Stable isotope ratios are expressed in per mil (‰) in delta (δ) notation using the following equation: $\delta X = [(R_{sample}/R_{standard}) - 1]$, where X is ^{13}C or ^{15}N and R equals $^{13}C/^{12}C$ or $^{15}N/^{14}N$. The standard materials for $\delta^{13}C$ and $\delta^{15}N$ values are Pee Dee Belemnite and atmospheric nitrogen, respectively. The precision, assessed by the standard deviation of replicate analyses of two standards, National Institute for Standards and Technology (NIST. Gaithersburg, MD, USA) 8414 and internal lab standard tilapia muscle (n = 69 for all), was <0.2‰ for $\delta^{15}N$ values and <0.1‰ for $\delta^{13}C$ values for both standards. The instrumentation accuracy checked throughout the period of time that these samples were analyzed was based on NIST standards 8573, 8547 and 8548 for $\delta^{15}N$ values and 8542, 8573 and 8574 for $\delta^{13}C$ values (n = 55 for all). The mean differences from the certified values were ≤0.2‰ for $\delta^{15}N$ values and ≤0.1‰ for $\delta^{13}C$ values.

Statistical analysis

Linear regressions were used to determine the effects of tissue decomposition over time on lipid-extracted $\delta^{13}C$, %C, $\delta^{15}N$ and %N values for each species. Exponential regressions were considered but did not result in a better model fit probably due to isotopic steady-state not being reached over the study duration. Analysis of variance (ANOVA) was used to determine significant differences in BULK $\delta^{13}C$ and $\delta^{15}N$ values between groups (days 0, 128 and 700) in the frozen long-term for each species. Paired t-tests were used to determine differences between open and closed vial treatments during tissue decomposition. The $\delta^{13}C$ and $\delta^{15}N$ values, and carbon and nitrogen content for each species and treatment, had a normal distribution based on visual inspection of histograms. Statistical analyses were performed in R version 3.2.4[19] with an α value of 0.05.

RESULTS

Frozen long-term storage treatment

The differences between the control and days 128 and 700 treatment samples for each species were minimal and similar to the measurement error for the mass spectrometer (0.2‰ for both $\delta^{13}C$ and $\delta^{15}N$ values (Table 1). There was no significant difference between the three time-interval

Rapid Communications in Mass Spectrometry

groups for $\delta^{13}C$ and $\delta^{15}N$ values in Greenland sharks ($\delta^{13}C$: $F_{1,11} = 0.09$, $p = 0.77$; $\delta^{15}N$: $F_{1,11} = 4.47$, $p = 0.06$), lake trout ($\delta^{13}C$: $F_{1,11} = 3.15$, $p = 0.10$; $\delta^{15}N$: $F_{1,11} = 0.01$, $p = 0.99$) and ringed seals ($\delta^{13}C$: $F_{1,11} = 2.25$, $p = 0.16$; $\delta^{15}N$: $F_{1,11} = 0.40$, $p = 0.54$).

Tissue decomposition treatment

The effects of muscle tissue decomposition on lipid-extracted $\delta^{13}C$, %C, $\delta^{15}N$, %N and C:N values varied between open and closed vial treatments amongst and within species (Table 2).

Table 1. Mean ± SD of $\delta^{13}C$ (‰), %C, $\delta^{15}N$ (‰), %N and C:N values from non-lipid-extracted (i.e. BULK) ringed seal, lake trout and Greenland shark muscle samples from day 0, 128 and 700 ($n = 3$ for each day). The effects of tissue preservation through freezing on $\delta^{13}C$ and $\delta^{15}N$ were assessed at day 128 and 700 for all species

Day	$\delta^{13}C$ (‰)	C (%)	$\delta^{15}N$ (‰)	N (%)	C:N
Ringed seal					
0	–18.0 ± 0.1	47.2 ± 0.2	15.3 ± 0.2	12.9 ± 0.3	3.7 ± 0.1
Frozen-128	–17.8 ± 0.1	45.0 ± 0.8	15.5 ± 0.2	12.8 ± 0.2	3.5 ± 0.1
Frozen-700	–17.8 ± 0.1	46.9 ± 1.6	15.5 ± 0.1	13.3 ± 0.5	3.5 ± 0.1
Lake trout					
0	–23.2 ± 0.4	47.7 ± 1.0	18.4 ± 0.2	12.1 ± 0.3	3.9 ± 0.2
Frozen-128	–23.4 ± 0.2	46.3 ± 0.7	18.6 ± 0.1	11.8 ± 0.2	3.9 ± 0.1
Frozen-700	–23.7 ± 0.5	47.0 ± 1.3	18.5 ± 0.1	11.5 ± 0.7	4.1 ± 0.3
Greenland shark					
0	–22.9 ± 0.1	62.5 ± 0.6	16.4 ± 0.1	5.3 ± 0.2	11.8 ± 0.4
Frozen-128	–22.8 ± 0.1	59.8 ± 0.4	16.6 ± 0.2	5.8 ± 0.3	10.2 ± 0.6
Frozen-700	–22.9 ± 0.2	59.0 ± 1.6	16.7 ± 0.2	5.6 ± 0.3	10.6 ± 0.7

Table 2. Mean ± SD of $\delta^{13}C$ (‰), %C, $\delta^{15}N$ (‰), %N and C:N values from ringed seal, lake trout and Greenland shark lipid-extracted muscle over decomposition time by open and closed lid treatment methods ($n = 3$ for each day by treatment method)

	Open lid					Closed lid				
Day	$\delta^{13}C$ (‰)	C (%)	$\delta^{15}N$ (‰)	N (%)	C:N	$\delta^{13}C$ (‰)	C (%)	$\delta^{15}N$ (‰)	N (%)	C:N
Ringed seal										
0	–18.2 ± 0.2	46.6 ± 0.1	15.3 ± 0.2	13.7 ± 0.1	3.4 ± 0.1	–18.2 ± 0.2	46.6 ± 0.1	15.3 ± 0.2	13.7 ± 0.1	3.4 ± 0.1
1	–18.1 ± 0.1	46.8 ± 0.4	15.7 ± 0.1	13.9 ± 0.1	3.4 ± 0.1	–18.1 ± 0.1	47.0 ± 0.4	15.8 ± 0.1	13.9 ± 0.1	3.4 ± 0.1
2	–18.2 ± 0.1	46.5 ± 0.2	15.6 ± 0.1	13.7 ± 0.1	3.4 ± 0.1	–18.0 ± 0.1	47.1 ± 0.1	15.5 ± 0.1	14.1 ± 0.1	3.4 ± 0.1
4	–18.4 ± 0.1	45.6 ± 0.4	15.5 ± 0.1	13.5 ± 0.1	3.4 ± 0.1	–18.2 ± 0.1	46.3 ± 0.3	15.3 ± 0.1	14.0 ± 0.1	3.3 ± 0.1
8	–18.6 ± 0.1	45.0 ± 0.2	15.6 ± 0.1	13.1 ± 0.1	3.4 ± 0.1	–18.2 ± 0.1	46.3 ± 0.6	15.7 ± 0.1	13.9 ± 0.2	3.3 ± 0.1
16	–18.4 ± 0.1	45.4 ± 0.2	15.6 ± 0.2	13.5 ± 0.1	3.4 ± 0.1	–18.1 ± 0.1	46.5 ± 0.3	15.7 ± 0.1	14.1 ± 0.1	3.3 ± 0.1
32	–18.4 ± 0.1	45.0 ± 0.5	15.6 ± 0.1	13.3 ± 0.3	3.4 ± 0.1	–18.0 ± 0.1	45.9 ± 0.4	16.0 ± 0.2	13.7 ± 0.1	3.4 ± 0.1
64	–18.5 ± 0.1	44.3 ± 0.2	15.8 ± 0.1	13.3 ± 0.1	3.3 ± 0.1	–17.8 ± 0.1	45.0 ± 0.5	16.1 ± 0.3	13.7 ± 0.2	3.3 ± 0.1
128	–18.1 ± 0.2	43.8 ± 2.7	15.8 ± 0.2	13.3 ± 0.8	3.3 ± 0.1	–18.0 ± 0.2	44.3 ± 0.4	16.2 ± 0.7	13.5 ± 0.2	3.3 ± 0.1
256	–17.9 ± 0.5	44.9 ± 0.1	16.0 ± 0.5	13.6 ± 0.1	3.3 ± 0.1	–17.5 ± 0.3	43.0 ± 0.3	17.5 ± 1.2	13.3 ± 0.1	3.2 ± 0.1
Lake trout										
0	–22.2 ± 0.1	46.3 ± 0.1	18.9 ± 0.1	14.1 ± 0.1	3.3 ± 0.1	–22.2 ± 0.1	46.3 ± 0.1	18.9 ± 0.1	14.1 ± 0.1	3.3 ± 0.1
1	–22.2 ± 0.1	45.7 ± 0.3	18.6 ± 0.1	14.1 ± 0.1	3.2 ± 0.1	–22.3 ± 0.1	46.0 ± 0.3	18.7 ± 0.1	14.2 ± 0.1	3.3 ± 0.1
2	–22.2 ± 0.1	44.6 ± 0.2	18.7 ± 0.1	13.7 ± 0.1	3.3 ± 0.1	–22.2 ± 0.1	45.2 ± 1.2	18.8 ± 0.2	14.0 ± 0.4	3.2 ± 0.1
4	–22.5 ± 0.1	43.7 ± 1.1	19.0 ± 0.2	13.1 ± 0.3	3.3 ± 0.1	–22.4 ± 0.1	44.9 ± 0.4	18.8 ± 0.2	13.7 ± 0.1	3.3 ± 0.1
8	–22.3 ± 0.2	43.0 ± 0.6	19.3 ± 0.3	12.9 ± 0.1	3.3 ± 0.1	–22.3 ± 0.2	42.9 ± 1.4	18.9 ± 0.1	13.1 ± 0.3	3.3 ± 0.1
16	–22.5 ± 0.2	42.8 ± 0.9	19.0 ± 0.2	12.9 ± 0.2	3.3 ± 0.1	–22.2 ± 0.2	42.7 ± 1.2	19.0 ± 0.1	12.9 ± 0.4	3.3 ± 0.1
32	–22.5 ± 0.2	43.8 ± 0.6	19.4 ± 0.2	12.9 ± 0.1	3.4 ± 0.1	–22.0 ± 0.3	40.6 ± 2.1	19.7 ± 1.3	12.4 ± 0.5	3.3 ± 0.1
64	–22.6 ± 0.3	42.8 ± 1.7	19.4 ± 0.1	12.8 ± 0.5	3.3 ± 0.1	–21.6 ± 0.3	41.8 ± 0.7	19.6 ± 0.5	13.0 ± 0.1	3.2 ± 0.1
128	–22.7 ± 0.2	42.7 ± 0.3	19.3 ± 0.2	13.0 ± 0.1	3.3 ± 0.1	–21.6 ± 0.1	39.8 ± 3.0	18.7 ± 0.1	12.7 ± 0.4	3.1 ± 0.1
256	–22.5 ± 0.2	42.5 ± 0.6	19.2 ± 0.1	12.9 ± 0.2	3.3 ± 0.1	–21.8 ± 0.2	39.0 ± 0.9	19.7 ± 0.1	12.2 ± 0.2	3.2 ± 0.1
Greenland shark										
0	–18.5 ± 0.2	46.8 ± 0.6	16.2 ± 0.1	13.4 ± 0.3	3.5 ± 0.1	–18.5 ± 0.2	46.8 ± 0.6	16.2 ± 0.1	13.4 ± 0.3	3.5 ± 0.1
1	–18.4 ± 0.1	46.5 ± 0.1	16.3 ± 0.2	13.5 ± 0.1	3.4 ± 0.1	–18.5 ± 0.3	47.1 ± 0.8	16.4 ± 0.2	13.5 ± 0.1	3.5 ± 0.1
2	–18.3 ± 0.1	46.0 ± 0.3	16.3 ± 0.2	13.5 ± 0.1	3.4 ± 0.1	–18.4 ± 0.1	46.6 ± 0.6	16.4 ± 0.1	13.5 ± 0.3	3.5 ± 0.1
4	–18.5 ± 0.1	47.0 ± 1.3	16.2 ± 0.1	13.5 ± 0.1	3.5 ± 0.1	–18.6 ± 0.1	47.4 ± 0.6	16.3 ± 0.2	13.3 ± 0.3	3.6 ± 0.1
8	–18.6 ± 0.1	45.7 ± 0.6	16.1 ± 0.2	13.2 ± 0.4	3.5 ± 0.1	–18.5 ± 0.6	46.4 ± 0.4	16.6 ± 0.4	13.0 ± 0.1	3.6 ± 0.1
16	–18.1 ± 0.2	44.8 ± 1.0	16.4 ± 0.3	13.4 ± 0.1	3.4 ± 0.1	–18.1 ± 0.4	44.8 ± 0.7	16.9 ± 0.3	12.8 ± 0.3	3.5 ± 0.1
32	–18.4 ± 0.1	45.8 ± 0.1	16.4 ± 0.1	13.6 ± 0.1	3.4 ± 0.1	–18.8 ± 0.2	46.1 ± 0.5	16.8 ± 0.1	12.8 ± 0.2	3.6 ± 0.1
64	–18.2 ± 0.2	45.8 ± 0.5	16.3 ± 0.2	13.8 ± 0.2	3.3 ± 0.1	–18.6 ± 0.1	45.6 ± 0.6	16.8 ± 0.1	13.0 ± 0.1	3.5 ± 0.1
128	–18.3 ± 0.3	45.9 ± 0.7	16.7 ± 0.2	13.7 ± 0.1	3.4 ± 0.1	–17.9 ± 0.1	43.2 ± 1.4	17.0 ± 0.4	12.7 ± 0.6	3.4 ± 0.1
256	–19.3 ± 0.1	43.5 ± 0.5	16.6 ± 0.1	11.9 ± 0.2	3.7 ± 0.1	–18.4 ± 0.1	43.0 ± 0.5	17.5 ± 0.5	12.1 ± 0.3	3.6 ± 0.1

Generally, the $\delta^{13}C$ and $\delta^{15}N$ values were significantly higher in the open vial treatment than in the closed vial treatment over 256 days across species (t_{29} = 2.53 – 5.73, all $p \leq 0.02$), except for the $\delta^{13}C$ values in Greenland sharks (t_{29} = 0.38, p = 0.71) and the $\delta^{15}N$ values in lake trout (t_{29} = 0.03, p = 0.97). There was no significant relationship between $\delta^{13}C$ values and decomposition time for the closed lid treatment in Greenland shark muscle (t_{28} = 1.35, p = 0.19) over 256 days. Generally, the $\delta^{13}C$ and $\delta^{15}N$ values significantly increased with decomposition time across treatments and species (t_{28} = 2.51–4.40, all $p < 0.02$), except for the $\delta^{13}C$ values of lake trout and Greenland shark muscle in the open vial treatment where a significant decrease occurred (t_{28} = –7.23 to –2.11, all $p < 0.04$; Fig. 1) over 256 days. Despite significant changes in the $\delta^{13}C$ and $\delta^{15}N$ values with decomposition time over 256 days across treatments and species, the magnitude of change up to 8 days was low and not significant (t_{13} = –2.06 to 1.81, all $p \geq 0.06$) with changes primarily being ≤0.1‰ for both $\delta^{13}C$ and $\delta^{15}N$ values (Table 2). However, changes were significant for ringed seal $\delta^{13}C$ values (t_{13} = –5.97, $p < 0.001$) and lake trout $\delta^{15}N$ values (t_{13} = 3.81, $p < 0.01$) in the open vial treatment over 8 days, although, after 16 days, the $\delta^{15}N$ values in lake trout were similar to that in the control (within 0.1‰; t_{16} = 1.88, p = 0.08). After 16 days, the magnitude of change was higher with changes primarily being ≤0.4‰ for $\delta^{13}C$ and $\delta^{15}N$ values (Table 2). After 32 days, changes in $\delta^{13}C$ values were still ≤0.3‰ but were higher for $\delta^{15}N$ values with changes generally being ≥0.3‰ and up to 0.7‰ (Figs. 1 and 2; Table 2). After 128 and 256 days, a higher magnitude of change occurred in $\delta^{15}N$ values (generally ≥0.5‰ up to 2.2‰) than in $\delta^{13}C$ values (generally ≥0.3‰ up to 0.8‰) across treatments and species where the largest changes occurred in the closed vial treatment (2.2‰ for ringed seals, 1.3‰ for Greenland sharks and 1.0‰ for lake trout; Figs. 1 and 2; Table 2). Overall, increases in $\delta^{13}C$ and $\delta^{15}N$ values and decreases in %C and %N were more pronounced in the closed vial than in the open vial treatment (Figs. 1 and 2; Table 2).

The variabilities (i.e. standard deviation) in $\delta^{13}C$ and $\delta^{15}N$ values after 128 and 256 days were all generally <0.2‰, similar to the observed variability at day 0, except for the ringed seal $\delta^{13}C$ and $\delta^{15}N$ values for both treatments (range = 0.3–1.2‰) and the Greenland shark $\delta^{15}N$ values in the closed vial treatment (0.5‰; Table 2). The carbon (%C) and nitrogen (%N) content significantly decreased for each

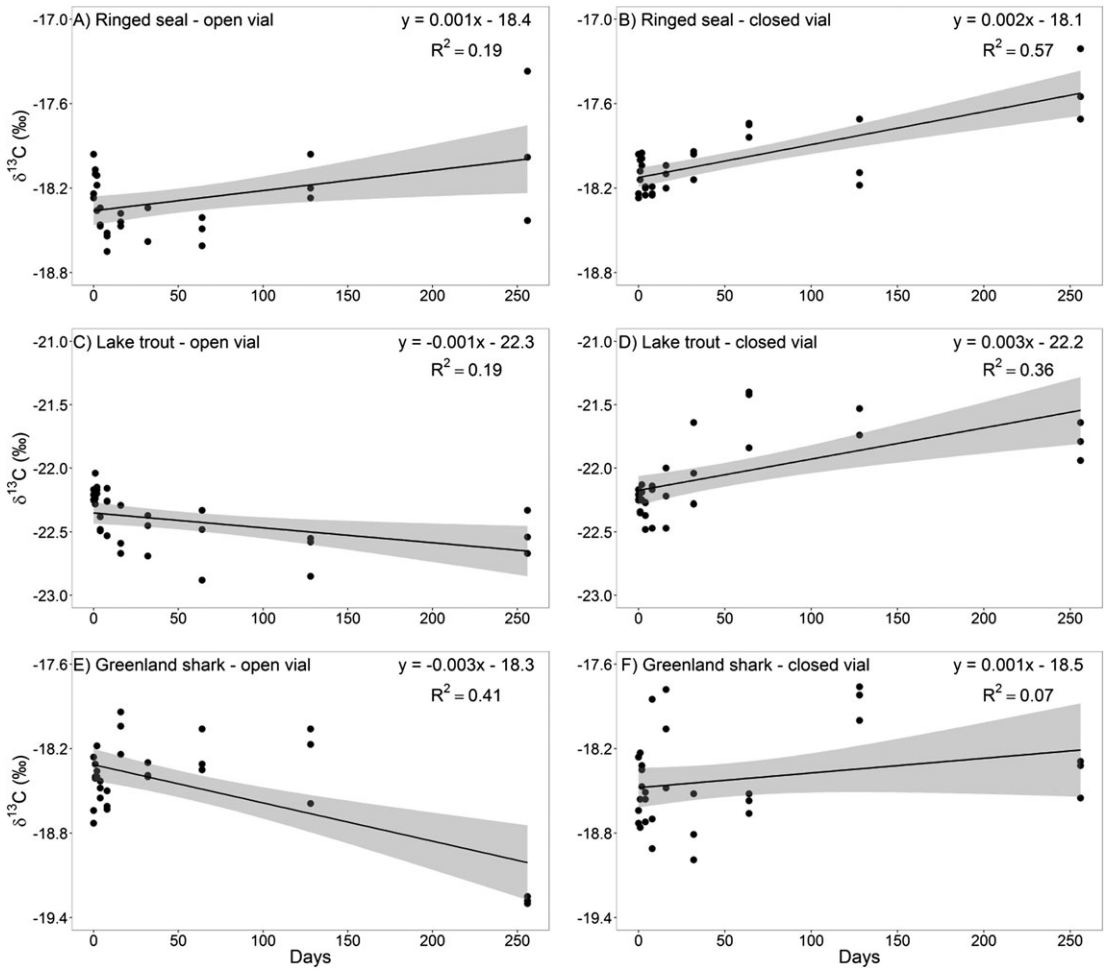

Figure 1. Linear regressions of the effects of decomposition under two different treatments (vial open and closed) on $\delta^{13}C$ (‰) values over time for ringed seal, lake trout and Greenland shark muscle samples. Gray bars represent the 95% confidence interval.

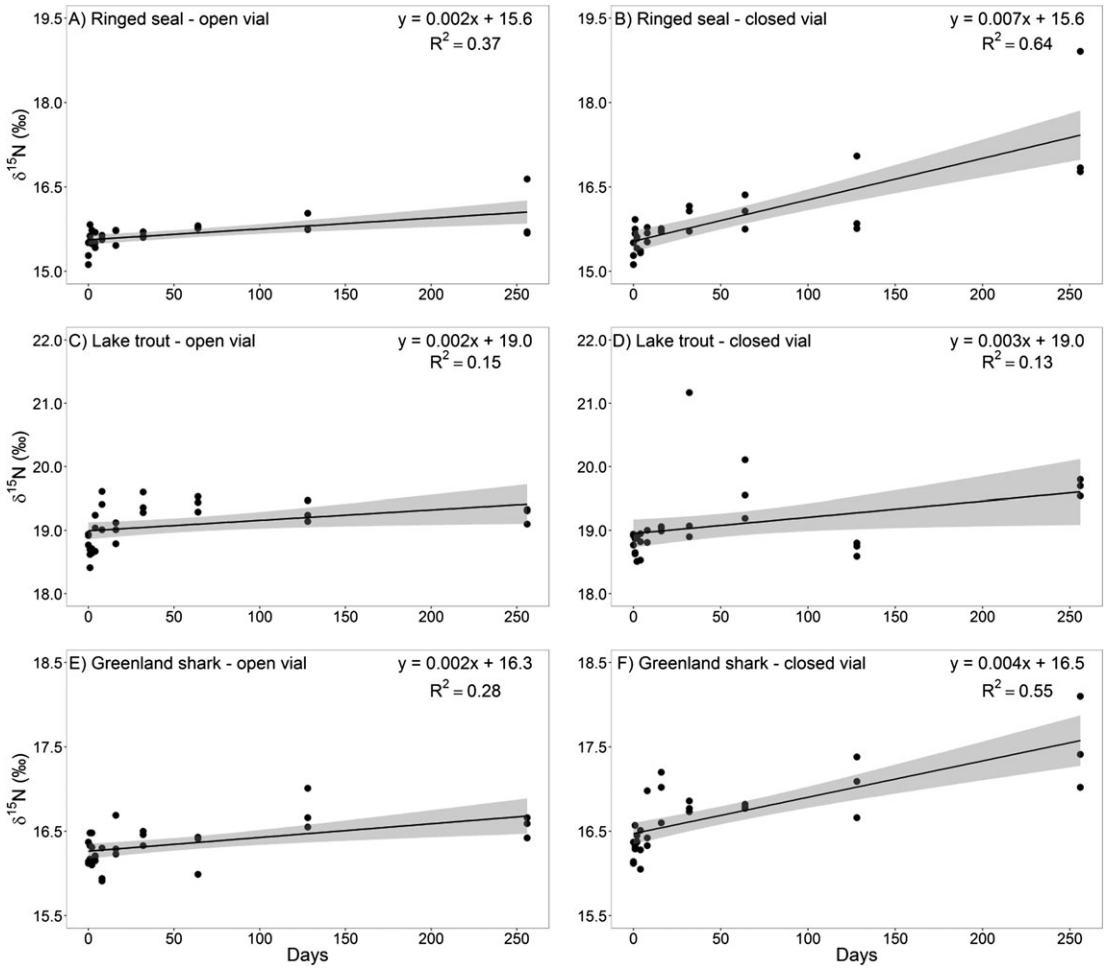

Figure 2. Linear regressions of the effects of decomposition under two different treatments (vial lid open and closed) on δ^{15}N (‰) values over time for ringed seal, lake trout and Greenland shark muscle samples. Gray bars represent the 95% confidence interval.

species and treatment over decomposition time (t_{28} = –14.22 to –2.34, all $p < 0.03$), except for the open vial treatment in ringed seals (t_{28} = –0.45, $p = 0.66$). Higher discrepancies between day 256 and day 0 in %C and %N occurred in lake trout (range = 8.2–15.8%) and Greenland sharks (7.0–9.7%) than in ringed seals (3.6–7.7%), and for the closed vial treatment (2.9 – 15.8%) than for the open vial treatment (3.6–8.5%) across species (Table 2).

DISCUSSION

This is the first study to examine the effects of freezing and decomposition on the δ^{13}C and δ^{15}N values of animal tissue over such extended treatment periods (700 and 256 days, respectively). Similar to previous studies, the effect of long-term storage of animal tissues in freezers on δ^{13}C and δ^{15}N values was negligible, even after 700 days, highlighting its use as a reliable storage method.[8,9,12] Surprisingly, despite significant relationships between δ^{13}C and δ^{15}N values and decomposition time across species and treatments, the magnitude of change up to 8 days was generally small for both δ^{13}C and δ^{15}N values (≤0.1‰) and similar to our instrument error, leading to reliable δ^{13}C and δ^{15}N values for ecological interpretations. However, changes in δ^{13}C and δ^{15}N values were higher after 8 days, identifying that caution should be used when using highly decomposed tissue for stable isotope analysis in ecological studies.

Frozen long-term storage treatment

Freezing is a commonly applied tissue preservation method by stable isotope ecologists, particularly for longitudinal studies that use archived samples stored for many years to address research questions. The δ^{13}C and δ^{15}N values of ringed seal, lake trout and Greenland shark muscle samples were not affected by the method of freezing as a long-term preservation technique over 700 days, similar to studies of relatively shorter duration ranging from days and weeks[6,20,21] to 21 months.[8] This is in contrast to Feuchtmayr and Grey[22] who reported a ^{13}C-depletion in zooplankton tissue due to a loss of carbon components[23] and mechanical breakdown of cells via leaching from thawing or filtering during their preparation procedure. Similarly, Barrow *et al.*[24] found a significant change in the δ^{13}C values of epidermis tissue from three turtle species when stored in freezers at –10°C after 30 days, but the magnitude of change was small (<0.2‰) and comparable

with their analytical variability (0.1‰). Similar to conclusions from other studies, we support the method of freezing animal tissue for extended time periods as a preservation technique to ensure the integrity of $\delta^{13}C$ and $\delta^{15}N$ values for ecological interpretations.

Tissue decomposition treatment

The effect of tissue decomposition on the stable isotope ratios of animal tissues has not been widely investigated. Here, tissue decomposition significantly enriched muscle tissue in both ^{13}C and ^{15}N for ringed seals, lake trout and Greenland sharks over the course of the study period. Previous experimental work found that small, yet significant changes in magnitude occurred in $\delta^{13}C$ values (0.7‰) compared with $\delta^{15}N$ values (0.4‰) between the control and experimental treatment samples from *Drosophila melanogaster* left to decompose for 10 days.[11] In contrast, the magnitude of change was much higher in killer whale (*Orcinus orca*) skin where the $\delta^{13}C$ values increased by 1.0‰ and the $\delta^{15}N$ values by 6.4‰ when left to decompose for 14 days.[12] Our results showing negligible changes in the $\delta^{13}C$ and $\delta^{15}N$ values of decomposing tissue over a similar time-frame (i.e. after 8 days) were more comparable to Ponsard and Amlou[11] than to Burrows *et al*.[12] The taxa- and tissue-specific effects of tissue decomposition on $\delta^{13}C$ and $\delta^{15}N$ values could be attributed to differences in structural proteins, such as collagen fibers, of the extracellular matrix but this requires further investigation.

Significant decreases in carbon (%C) and nitrogen (%N) content and no change in C:N with increasing decomposition time probably indicated biodegradation where microorganisms likely metabolized ringed seal, lake trout and Greenland shark muscle into simpler waste components. Due to a kinetic isotope effect and isotope fractionation, microorganisms preferentially uptake the lighter isotopes of muscle tissue over decomposition time and discriminate against the heavier isotopes, which is a commonly observed phenomenon in microbial ecology.[25,26] Our results are consistent with this proposed mechanism as ringed seal, lake trout and Greenland shark muscle was more enriched in ^{13}C and ^{15}N with increasing decomposition time relative to the control. Overall, the enrichment in ^{13}C and ^{15}N of animal tissue up to 8 days was minimal and not significant; therefore, stable isotope analysis of rotting muscle tissue up to approximately a week is still reliable for ecological studies examining consumer diet, food web structure, carbon sources and animal movements.

Air and water availability is an important environmental factor influencing microbial numbers and activities.[27] In this study, the carbon and nitrogen content, and the $\delta^{13}C$ and $\delta^{15}N$ values of muscle across species, changed at faster rates in the closed vial treatment that would have more moisture and less air availability than the open vial treatment. This has implications for the preservation of muscle samples which are typically stored in closed vials or in sealed sterilized plastic bags within coolers with dry ice or freeze-packs when in a shipment delay, as well as after proper tissue storage with laboratory freezer breakdowns. When muscle tissue has thawed, it has up to 8 days to then be stored frozen before the integrity of the $\delta^{13}C$ and $\delta^{15}N$ values becomes jeopardized.

Lipids can act as a preservative agent for muscle tissue by providing an oxygen or moisture barrier due to their hydrophobicity and tightly packed crystalline structure.[16] Ringed seals had low lipid content in their muscle tissue as their blubber layer is primarily used for lipid energy storage, whereas, in temperate and polar fish species, lipid stores in muscle are presumed to provide calorific stores.[28] Higher lipid content in fish muscle occurs in polar regions,[29] and in the case of the Greenland shark, may aid in regulating buoyancy.[30] Despite these species-specific differences in lipid content, the $\delta^{13}C$ and $\delta^{15}N$ values changed similarly across species, suggesting that the presence of lipids did not preserve the integrity of the $\delta^{13}C$ and $\delta^{15}N$ values for any species over the study period. However, differences in the amounts of lipid types such as storage (i.e. fatty acids) and structural (i.e. phospholipids) between species could affect this interpretation and this requires further investigation. In addition, lipid extraction removes cell membrane proteins and nitrogenous waste products[31] which can slightly alter $\delta^{15}N$ values by 0.2 or 0.3‰ in these species.[13,15] This would have a negligible effect on interpretations from our study due to the use of homogenized samples from one individual per species and the higher magnitude of change occurring with tissue decomposition (≥0.3‰) than by lipid extraction.

Investigating the causative mechanisms of animal tissue decomposition on $\delta^{13}C$ and $\delta^{15}N$ values was beyond the scope of this study. Our results showed that tissue decomposition alters $\delta^{13}C$ and $\delta^{15}N$ values, albeit the changes were slight, and that species-specific differences in lipid content did not preserve the integrity of $\delta^{13}C$ and $\delta^{15}N$ values over time. Our analyses were performed solely on muscle tissue, and, although this is commonly used in isotopic studies,[14] numerous other tissues such as blood, plasma, liver and skin are also routinely used. Further investigation into taxa- and tissue-specific effects of rotting animal tissue on $\delta^{13}C$ and $\delta^{15}N$ values should be performed, as discrepancies over its effects on $\delta^{13}C$ and $\delta^{15}N$ values have been observed between invertebrates and vertebrates[11,12] and between skin and muscle of aquatic vertebrates.[12]

CONCLUSIONS

We highlight: (1) long-term storage (up to 2 years) of animal tissue in freezers at –20°C preserves the integrity of $\delta^{13}C$ and $\delta^{15}N$ values for subsequent analysis; (2) tissue lipid content did not aid in preserving the validity of $\delta^{13}C$ and $\delta^{15}N$ values of animal tissue with decomposition time at room temperature; and (3) tissue decomposition significantly decreased %C and %N and enriched animal tissue in ^{13}C and ^{15}N over the study period, although the changes in magnitude were negligible up to 8 days. Thus, the $\delta^{13}C$ and $\delta^{15}N$ values derived from tissue rotting for a week are reliable. We therefore recommend that tissue from recently stranded or deceased animals that has been partially decomposed is useable for stable isotope analysis. In addition, there should be minimal effect on the $\delta^{13}C$ and $\delta^{15}N$ values of muscle tissue that is not immediately stored frozen following field sampling and tissue where freezer breakdown has occurred and samples may have thawed for a short period (i.e. days). However, after weeks, the $\delta^{13}C$ and $\delta^{15}N$ values could be compromised from highly decomposed tissue.

Acknowledgements

We thank E. Mailloux for her assistance in sample preparation. We thank three anonymous reviewers whose comments improved this manuscript. This study was supported by funding from NSERC Discovery to ATF. DJY was supported by funding from University of Windsor, Ontario Graduate Scholarship and the W. Garfield Weston Foundation.

REFERENCES

[1] M. J. DeNiro, S. Epstein. Influence of diet on the distribution of carbon isotopes in animals. *Geochim. Cosmochim. Acta* **1978**, *42*, 495.
[2] C. A. Layman, M. S. Araujo, R. Boucek, C. M. Hammerschlag-Peyer, E. Harrison, Z. R. Jud, P. Matich, A. E. Rosenblatt, J. J. Vaudo, L. A. Yeager, D. M. Post, S. Bearhop. Applying stable isotopes to examine food-web structure: an overview of analytical tools. *Biol. Rev.* **2011**, *87*, 545.
[3] B. J. Peterson, B. Fry. Stable isotopes in ecosystem studies. *Annu. Rev. Ecol. Syst.* **1987**, *18*, 293.
[4] R. L. France. Carbon-13 enrichment in benthic compared to planktonic algae: foodweb implications. *Mar. Ecol. Prog. Ser.* **1995**, *124*, 307.
[5] M. J. DeNiro, S. Epstein. Influence of diet on the distribution of nitrogen isotopes in animals. *Geochim. Cosmochim. Acta* **1981**, *45*, 341.
[6] S. Kaehler, E. A. Pakhamov. Effects of storage and preservation on the $\delta^{13}C$ and $\delta^{15}N$ signatures of selected organisms. *Mar. Ecol. Prog. Ser.* **2001**, *219*, 299.
[7] H. C. Sarkinos, M. L. Johnson, M. J. Vander Zanden. A synthesis of tissue-preservation effects on carbon and nitrogen stable isotope signatures. *Can. J. Zool.* **2002**, *80*, 381.
[8] C. J. Sweeting, N. V. C. Polunin, S. Jennings. Tissue and fixative dependent shifts of $\delta^{13}C$ and $\delta^{15}N$ in preserved ecological material. *Rapid Commun. Mass Spectrom.* **2004**, *18*, 2587.
[9] K. A. Hobson, H. L. Gibbs, M. L. Gloutney. Preservation of blood and tissue samples for stable-carbon and stable-nitrogen isotope analysis. *Can J. Zool.* **1997**, *75*, 1720.
[10] A. M. de Lecea, A. J. Smit, S. T. Fennessy. The effects of freeze/thaw periods and drying methods on isotopic and elemental carbon and nitrogen in marine organisms, raising questions on sample preparation. *Rapid Commun. Mass Spectrom.* **2011**, *25*, 3640.
[11] S. Ponsard, M. Amlou. Effects of several preservation methods on the isotopic content of Drosophila samples. *C. R. Acad. Sci. III* **1999**, *322*, 35.
[12] D. G. Burrows, W. L. Reichart, M. B. Hanson. Effects of decomposition and storage conditions on the $\delta^{13}C$ and $\delta^{15}N$ isotope values of killer whale (*Orcinus orca*) skin and blubber tissue. *Mar. Mamm. Sci.* **2014**, *30*, 747.
[13] N. E. Hussey, J. A. Olin, M. J. Kinney, B. C. McMeans, A. T. Fisk. Lipid extraction effects on stable isotope values ($\delta^{13}C$ and $\delta^{15}N$) of elasmobranch muscle tissue. *J. Exp. Mar. Biol. Ecol.* **2012**, *434/435*, 7.
[14] D. M. Post, C. A. Layman, D. A. Arrington, G. Takimoto, J. Quattrochi, C. G. Montaña. Getting to the fat of the matter: models, methods and assumptions for dealing with lipids in stable isotope analysis. *Oecologia* **2007**, *152*, 179.
[15] D. J. Yurkowski, N. E Hussey, C. A. D. Semeniuk, S. H. Ferguson, A. T. Fisk. 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. *Pol. Biol.* **2015**, *38*, 131.
[16] C. N. Cutter. Opportunities for bio-based packaging technologies to improve the quality and safety of fresh and further processed muscle foods. *Meat Sci.* **2006**, *74*, 131.
[17] E. G. Bligh, W. J. Dyer. A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* **1959**, *37*, 911.
[18] B. C. McMeans, J. A. Olin, G. W. Benz. Stable-isotope comparisons between embryos and mothers of a placentatrophic shark species. *J. Fish Biol.* **2009**, *75*, 2464.
[19] R Core Team. *R: A language and environment for statistical computing*. R Foundation for Statistical Computing, Vienna, Austria, **2015**.
[20] K. L. Bosley, S. C. Wainright. Effects of preservatives and acidification on the stable isotope ratios (^{15}N:^{14}N, ^{13}C:^{12}C) of two species of marine animals. *Can. J. Fish. Aquat. Sci.* **1998**, *56*, 2181.
[21] M. L. Gloutney, K. A. Hobson. Field preservation techniques for the analysis of stable-carbon and nitrogen isotope ratios in eggs. *J. Field Ornithol.* **1998**, *69*, 223.
[22] H. Feuchtmayr, J. Grey. Effect of preparation and preservation procedures on carbon and nitrogen stable isotope determinations from zooplankton. *Rapid Commun. Mass Spectrom.* **2003**, *17*, 2605.
[23] K. Salonen, J Sarvala. The effect of different preservation methods on the carbon content of *Megacyclops gigas*. *Hydrobiologia* **1980**, *72*, 281.
[24] L. M. Barrow, K. A. Bjorndal, K. J. Reich. Effects of preservation method on stable carbon and nitrogen isotope values. *Physiol. Biochem. Zool.* **2008**, *81*, 688.
[25] M. J. Whiticar. Carbon and hydrogen isotope systematics of bacterial formation and oxidation of methane. *Chem. Geol.* **1999**, *161*, 291.
[26] S. Radajewski, P. Ineson, N. R. Parekh, J. C. Murrell. Stable-isotope probing as a tool in microbial ecology. *Nature* **2000**, *403*, 606.
[27] T. L. Kieft, P. S. Amy, F. J. Brockman, J. K. Fredrickson, B. N. Bjornstad, L. L. Rosacker. Microbial abundance and activities in relation to water potential in the vadose zones of arid and semiarid sites. *Microb. Ecol.* **1993**, *26*, 59.
[28] B. D. Sidell, E. L. Crockett, W. R. Driedzic. Anatarctic fish tissues preferentially catabolise monoeonic fatty acids. *J. Exp. Zool.* **2005**, *271*, 73.
[29] C. Friedrich, W. Hagen. Lipid contents of five species of notothenioid fish from high-Antarctic waters and ecological implications. *Polar Biol.* **1994**, *14*, 359.
[30] N. E. Hussey, M. A. MacNeil, J. A. Olin, B. C. McMeans, M. J. Kinney, D. D. Chapman, A. T. Fisk. Stable isotopes and elasmobranchs: tissue types, methods, applications and assumptions. *J. Fish Biol.* **2012**, *80*, 1449.
[31] A. T. Fisk, S. A. Tittlemier, J. L. Pranschke, R. J. Norstrom. Using anthropogenic contaminants and stable isotopes to assess the feeding ecology of Greenland sharks. *Ecology* **2002**, *83*, 2162.

