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NOTE

Long-term storage at –20°C compromises fatty acid composition of polar bear adipose biopsies

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ABSTRACT: This study aimed to gain insight into the influence of storage time and temperature on fatty acid (FA) signatures of biopsies of marine mammal adipose/blubber tissues. To examine storage effects, biopsy-type slices from larger pieces of adipose tissues from 2 polar bears *Ursus maritimus* were stored at either −20 or −80°C and subsequently analyzed for fatty acid composition initially (before storage), after 4 yr, and after 9 yr. At −20°C, after both 4 and 9 yr, proportions of polyunsaturated FAs significantly decreased, and proportions of monounsaturated FAs increased. Proportions of saturated FAs significantly increased only after 9 yr at −20°C in samples of 1 individual. After 4 and 9 yr of storage at −80°C, proportions of the 3 FA classes did not significantly change overall. Intra-individual differences in FA proportions increased over time in −20°C conditions, further pointing to biases stemming from inadequate storage conditions. These findings support the need to store biopsied fatty tissues (or other similarly thin and/or small adipose/blubber samples) at or below −80°C to adequately preserve FA signatures in samples over time for retrospective applications such as dietary studies.

KEY WORDS: Blubber · Polyunsaturated fatty acids · PUFA · Degradation · Frozen storage · *Ursus maritimus* · Feeding ecology · Diet · Marine mammal

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1. INTRODUCTION

Marine mammals serve as important biological indicators of environmental health and change, including monitoring climate-associated food web changes (McKinney et al. 2013, Boucher et al. 2019), foraging habitat changes (Stern et al. 2021), environmental pollution, and individual/population health (Moore 2008, Bossart 2011, Dietz et al. 2018). Yet, it remains challenging to assess fundamental aspects of marine mammal biology and ecology, given their aquatic and often remote habitats, as well as ethical, logistical, and financial constraints associated with collections of tissue samples from free-ranging populations (Noren & Mocklin 2012, Castrillon & Bengtson Nash 2020). Frequently, the only minimally invasive samples which can be collected from live individuals are small tissue biopsies, consisting of skin and an outer layer of blubber/adipose tissue. These biopsy samples have been used to understand feeding patterns using skin stable isotopes and adipose/blubber fatty acid (FA) signatures (Bowen & Iverson 2013, Bourque et al. 2018, Remili et al. 2023) and in relation to body condition measured as blubber thickness, adipocyte index, and/or lipid content (McKinney et al. 2014, Castrillon & Bengtson Nash 2020).

Although used to provide insight into many aspects of the biology and ecology of marine mammals, small

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tissue biopsies may be susceptible to degradation while in storage before analysis, or if archived samples are used for retrospective studies. Degradation may alter the FA signatures, impacting the ecological inferences drawn from those measurements (Budge et al. 2006, Lind et al. 2018). Few studies have examined the effects of storage conditions on the FA proportions of tissues over long periods, and those that have examined the issue in mammals or fish have reported variable results. In the blubber of Baltic grey seals *Halichoerus grypus* stored at −25°C, FA proportions remained largely unchanged over 6 yr (Lind et al. 2012), while in archived Baltic herring *Clupea harengus membras*, the proportions of multiple long-chain polyunsaturated FAs (PUFAs) decreased from 1980 to 2009 at −25°C (Lind et al. 2018). The relative influence of FA deterioration versus actual temporal declines in PUFAs in the population was not clear (Lind et al. 2018), although PUFAs are more susceptible to oxidation than saturated FAs (SFAs) and monounsaturated FAs (MUFAs) as they contain multiple unstable double bonds (Budge et al. 2006). Thus, understanding the effects of storage conditions on FA signatures in marine mammal tissues is required to confidently use these markers to answer ecological questions.

We assessed the influence of storage time and temperature on FA proportions in marine mammal fat storage (i.e. adipose, blubber) tissues using polar bears *Ursus maritimus*, a sentinel marine mammal species in the Arctic. To do so, we made biopsy-type slices from larger pieces of adipose tissue taken from 2 subsistence-harvested polar bears from West Greenland in 2011 and analyzed them for FA composition immediately after slicing (2012), after 4 yr of storage at −20 or −80°C (2016), and after 9 yr at −20 or −80°C (2021).

2. MATERIALS AND METHODS

2.1. Sample collection and sub-sampling

Full-depth adipose tissue (polar bear FA signatures do not vary with adipose depth; Thiemann et al. 2006) from 2 adult polar bears was collected in January and February 2011 by Thule subsistence hunters in West Greenland (Bear 1: ID #41447, female; Bear 2: ID #41452, male). After harvest, samples were shipped under storage at −20°C from Greenland to Denmark before being shipped on dry ice to Canada and later stored at −80°C prior to initial analysis in June 2012. Muscle and skin were removed, and the adipose tissue was scraped to reveal the freshest tissue, which was then sub-sampled into 15 full-depth slices (i.e. sampling from the outermost tissue closest to the skin through to the innermost tissue closest to muscle) for each bear and placed into clear glass vials (2 ml) with a Teflon-lined cap. At 16 mo after harvest, sub-samples were cut to reasonably replicate the size of a biopsy (0.01 to 0.05 g), which is more susceptible to oxidation than large pieces of tissue (Budge et al. 2006). Vials were not flushed with nitrogen before being capped. For each bear, 3 replicate adipose slices were analyzed under each of the following conditions (i.e. treatments): 'initially' (in 2012; 0Yr), after 4 yr of storage at −20°C (in 2016; 4Yr at −20°C) and −80°C (4Yr at −80°C), and after 9 yr at −20°C (in 2021; 9Yr at −20°C) and −80°C (9Yr at −80°C).

2.2. Fatty acid analysis

Prior to analysis, the oxidation of each sample was visually classified on a scale of 1 to 5 (1: white/fresh; 2: mainly white/slight yellow tinge; 3: white-yellow; 4: mainly yellow/slight white tinge; 5: yellow/sometimes a bit dry; McKinney et al. 2013). Samples were analyzed for FA signatures using established protocols (Budge et al. 2006, McKinney et al. 2013). Briefly, FAs were extracted using a modified Folch method and then trans-esterified with the Hilditch reagent to form fatty acid methyl esters (FAMEs). The FAMEs were separated and identified, and the mass percentage of each of 69 FAs was quantified by gas chromatography with flame ionization detection (GC-FID). Triplicate samples for each treatment and individual were run in 3 batches (2012, 2016, 2021). Pairwise differences between the triplicate samples were calculated, averaged, and reported as mean difference (%) for every FA subjected to each of the storage conditions. To assess accuracy, a standard reference material of whale blubber homogenate (NIST1945) was also extracted and run (n = 6). It showed a mean percent difference of 22% from previously published values (Kucklick et al. 2010).

2.3. Data analysis

The FAs were classified into 3 groups: SFAs (n = 10), MUFAs (n = 16), and PUFAs (n = 16). Only FAs in proportions greater than 0.1% were included in the analysis, to avoid undue influence of analytical variation related to small peaks. The normality and homoscedasticity of the data were checked using the Sha-

piro-Wilks test and diagnostic plots. A repeated-measures analysis of variance (ANOVA) was used to test for differences in overall FA proportions subjected to different treatments (0Yr, 4Yr at −20°C, 4Yr at −80°C, 9Yr at −20°C, and 9Yr at −80°C) for each FA group (SFA, MUFA, and PUFA) using the polar bear identity as a random factor. ANOVAs indicating significant differences within the 3 FA classes ($\alpha = 0.05$) were followed by a Dunnett's test to investigate differences between the FA proportions for each bear after being subjected to different storage conditions, compared to the control group (0Yr). Changes in individual FA proportions due to storage conditions were also tested using Dunnett's test. All statistical analyses were run in R 4.1.2, using the aov and DunnettTest functions (R Core Team 2021).

3. RESULTS

FA proportions of the triplicate samples varied within and between individuals (Table 1). Mean differences ranged from 4.3 to 20.8% and 4.2 to 11.8% between triplicates subjected to the same storage conditions for Bears 1 and 2, respectively. Samples stored at −20°C for 9 yr had the lowest precision for both bears. Bear 1 had similarly low precision at −80°C after 9 yr, but when 1 replicate was excluded, the mean difference was only 7.1%. Samples stored at −20°C for 4 and 9 yr had visibly degraded, but samples originating from the 2 individuals changed differently. Samples were visually degraded after 4 and 9 yr of storage at −20°C, but not at −80°C (Table 1, see the Supplement at www.int-res.com/articles/suppl/m728p075_supp.pdf).

There were significant differences between storage conditions for SFAs ($F_{4,24} = 11.1$, $p < 0.0001$), MUFAs ($F_{4,24} = 62.8$, $p < 0.0001$) and PUFAs ($F_{4,24} = 48.5$, $p < 0.0001$) compared to 0Yr FA proportions (Fig. 1, Table S1). After 9 yr at −20°C, samples from Bear 1 showed significantly higher SFA proportions than at 0Yr (Dunnett's test: $p < 0.001$). MUFA proportions increased significantly for both individuals in samples stored for 4 and 9 yr at −20°C (both bears: $p_{4Yr} < 0.001$, $p_{9Yr} < 0.0001$). PUFA proportions significantly decreased over 4 and 9 yr at −20°C for both individuals as well (Bear 1: $p_{4Yr} = 0.008$, $p_{9Yr} < 0.001$; Bear 2: $p_{4Yr} < 0.001$, $p_{9Yr} < 0.001$).

After 4 yr at −20°C, 3 MUFAs and 7 PUFAs significantly changed from 0Yr compositions, while at −80°C, only 1 FA in each class significantly changed. After 9 yr in −20°C conditions, 2 SFAs, 8 MUFAs, and 13 PUFAs significantly changed from 0Yr compositions, while in −80°C conditions, only 1 PUFA changed. For the individual FAs that did change with storage time and temperature, the mean proportions of most mirrored the change observed for their class, but a few changed differently than the trend observed for the class overall (Table S1). MUFAs overall increased in FA proportions; however, 14:1n5 displayed a decrease in proportions after 4 yr at −20°C ($p < 0.0001$) and −80°C ($p < 0.0001$) in samples from both bears. For PUFAs, most FAs significantly decreased (and for C20 and higher PUFAs, often fell to half or even less of their original values; Table S1), while 16:2n4 (−20°C: $p_{4Yr} = 0.016$; −80°C: $p_{4Yr} = 0.016$, $p_{9Yr} < 0.001$) and 20:2n9 (−20°C: $p_{4Yr} = 0.047$, $p_{9Yr} = 0.009$) increased in samples for both individuals while in storage.

Table 1. Subjective oxidation class (1= visibly fresh; 5 = visual degraded) and mean difference (%) in FA composition between triplicate samples for sets of 2 polar bear samples, according to storage time and conditions

Individual	Time (yr)	Temperature (°C)	Mean difference (%)	Oxidation class
Bear 1 (ID #41447)	0	–	4.3	2
	4	−20	14.5	4
	4	−80	6.5	2
	9	−20	20.8	4
	9	−80	20.7[a]	3
Bear 2 (ID #41452)	0	–	4.5	2
	4	−20	4.5	2
	4	−80	4.5	1
	9	−20	11.8	3
	9	−80	4.2	2

[a]The high mean difference at −80°C after 9 yr for Bear 1 was driven by 1 replicate from that treatment varying from the others. When excluded, the mean difference between Bear 1 replicates stored at −80°C for 9 yr is much lower (7.1%)

4. DISCUSSION

FA proportions were variable among treatments, as well as between individuals. The large mean difference between triplicate Bear 1 samples for 9Yr at −80°C is possibly due to an outlier, seemingly causing artificial variation between samples. We speculate that this sample may not have been capped as tightly or the cap may have loosened

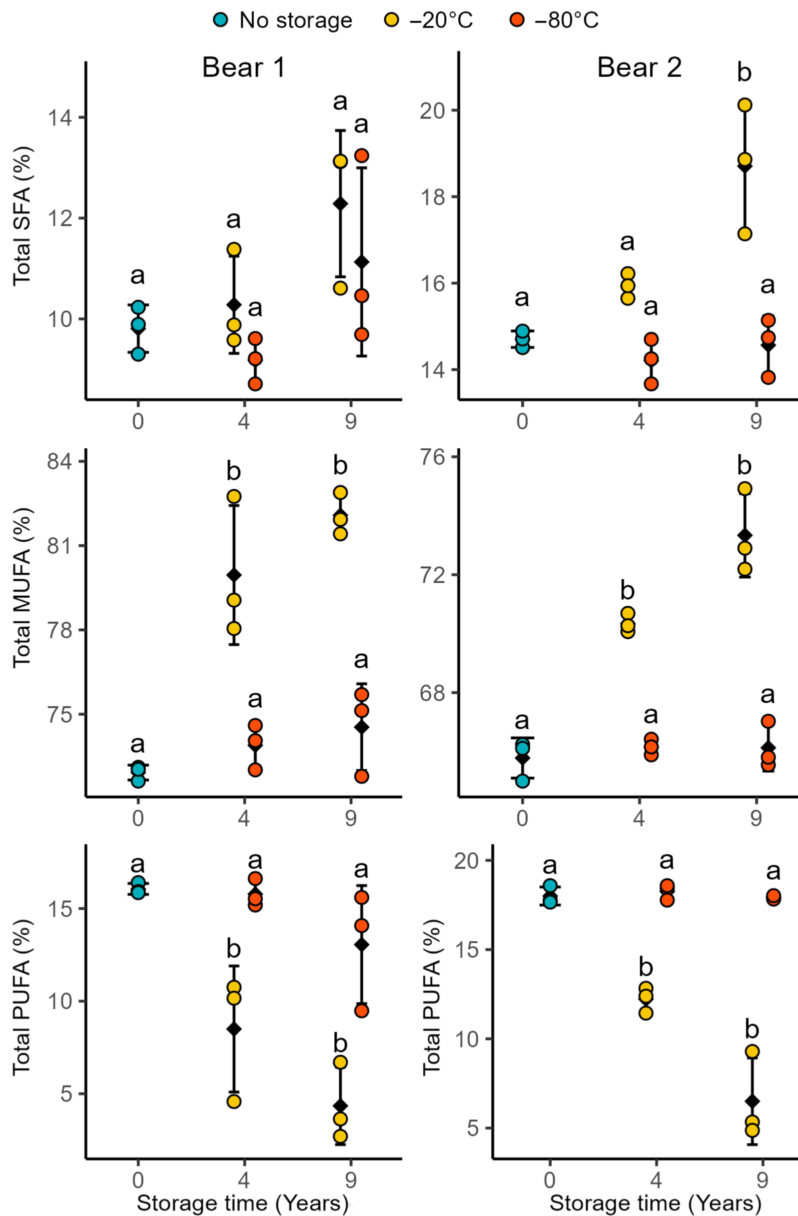

Fig. 1. Variation of total fatty acid (FA) proportions of the 3 structural groups (SFA: saturated FAs; MUFA: monounsaturated FAs; PUFA: polyunsaturated FAs) according to storage condition, for 2 polar bears. Points are colored according to storage temperature. Black diamonds: mean value for each treatment; error bars: SD; a: no significant difference in FA proportions between the treatment and the control '0Yr'; b: significant difference

during a lab move 6 yr into the experiment, and may have been subject to greater oxidation (Budge et al. 2006). Samples from Bear 2, conversely, showed good precision for all replicates stored at both −20 and −80°C over 4 and 9 yr. Samples stored at −80°C showed almost no intra-individual variation after 9 yr compared to those stored at −20°C over 9 yr.

The preservation of FA proportions in biopsy-type samples stored at −80°C for almost a decade was remarkably robust. Both individual polar bear samples showed minimal to no statistically significant changes in SFAs, MUFAs, and PUFAs under −80°C conditions. Only 2 FAs exhibited notable changes: 14:1n5 and 16:2n4 displayed significant differences from the initial samples. In other experiments, the sampling of inner sections of tissue allowed FA signatures in seal blubber stored at −25°C for 4 yr to remain unchanged (Lind et al. 2012), as well as FAs in mammalian tissues stored at −80°C, −20°C, and +20°C over a period of 6 mo (Nieminen et al. 2018). It is possible that the 'core' subsamples, sampled from tissue protected from air exposure, were protected from oxidation on all sides while the 'edge' subsamples, sampled from tissue exposed to air on 1 side, were protected on most sides. In the current study, as would be the case for marine mammal biopsies in general, it was not possible to subsample a 'core' section of the biopsies protected from air exposure, as biopsies are too thin and small to do so. Despite this, and the absence of nitrogen flushing of the sample vials (to reduce exposure to oxygen, recommended as best practice by Budge et al. 2006 and Nieminen et al. 2018), the −80°C storage effectively preserved FA signatures for almost a decade. The consistent results obtained without using these additional preservation measures strongly support the reliability of −80°C storage for long-term preservation of adipose biopsy samples.

It is possible that other degradation pathways besides oxidation resulted in changes in FA signatures after storage at −20°C. Enzymatic or microbial degradation occurring between the time the samples were harvested and the moment they were placed into frozen storage (Budge et al. 2006, Paczkowski & Schütz 2011) would have affected all samples equally and would have ceased once temperatures dropped below the optimal temperature range of the enzyme/bacteria (Budge et al. 2006, Paczkowski & Schütz 2011, Nieminen et al. 2018). The most likely cause of FA signature deterioration in our samples is thus

chemical oxidation (Budge et al. 2006), which generally occurs when lipid samples are in contact with oxygen in the air. In a study in which human adipose tissue aspirates were stored in Luer adapters, leaving only a small area of the samples exposed to air, Katan et al. (2003) found only minor decreases in omega-3 PUFA proportions when lipid samples were stored at room temperature for 6 yr. In our experimental setup, lipid samples were contained in relatively large containers compared to their size, leaving the entire surface exposed to air. Previous studies (Lind et al. 2012, Nieminen et al. 2018) posited that the storage temperature may not be an important factor in determining the stability of FA signatures. On the contrary, our study shows that, for small samples like biopsies, −80°C storage is critical, whereas it is probably less necessary if large tissue pieces (e.g. from subsistence-harvested individuals) can be obtained, from which inner tissue can be sub-sampled later, or the storage time at a higher temperature is on the order of months rather than years.

Highly unsaturated FAs with 20−22 carbon chain length, used to assess feeding and diet (Budge et al. 2006), seem to be most susceptible to degradation (Lind et al. 2018). Our results show excellent stability of PUFA proportions after almost a decade of storage at −80°C. Consequently, our results highlight that it is imperative that the adipose or blubber biopsy samples be kept in an ultra-cold freezer at −80°C to ensure the accuracy and reliability of FA-based diet studies. Inaccurate interpretation of dietary patterns and diet composition estimates based on FA signatures may occur with improperly stored adipose biopsies. In addition, it may be useful in follow-up work to investigate whether any significant FA degradation occurs during short-term storage during field collection.

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