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ORIGINAL PAPER

Factors affecting the degree of vertical stratification of fatty acids in grey seal blubber

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Abstract

The biochemistry of marine mammal blubber differs vertically from skin to muscle, which forms a challenge for using fatty acids (FAs) from differently sampled blubber as a proxy for dietary studies required for ecosystem-based management of coastal resources. In the blubber of some phocid seal individuals, the vertical stratification of several FAs is pronounced whereas in others the FAs distribute almost evenly through the blubber column. Using gas chromatography, we analysed the blubber vertical FA profiles of 30 adult male grey seals from the Baltic Sea, and examined which factors induced the largest vertical change of FA composition detected at the depth of 15–18 mm (outer and middle blubber boundaries). It was revealed that the degree of this compositional shift did not depend on the blubber thickness. Seal age only affected the vertical distribution of the FAs 16:0 and 16:1n-7. However, the outer blubber ratio of Δ9-desaturated monounsaturated FAs (MUFAs) to their saturated FA (SFA) precursors was not increased by grey seal age, contrasting earlier findings for ringed seals. A major determinant of the degree of FA stratification between the outer and middle blubber was the mismatch between the individually varying FA composition of the innermost blubber, regarded to reflect the dietary FA supply the most, and the uniform FA composition of endogenously regulated MUFA-rich outer blubber. Thus, discarding a fixed-depth layer of the grey seal outermost blubber, which we here show to span 0–18 mm from skin and which to a lesser extent reflects the diet of the individual, may in the case of small pinnipeds improve the sensitivity of the FA analysis in assessing spatial, temporal and individual dietary differences. When studying the outer blubber samples using only the diet-derived PUFA variables (SFAs and MUFAs omitted), the sensitivity of the analysis was better than when using this sample type with all main FA variables included.

Introduction

Studying blubber fatty acid (FA) composition is a frequently used method to monitor the diet of marine mammals (e.g. Bradshaw et al. 2003; Walton and Pomeroy 2003; Budge et al. 2006; Guerrero et al. 2016; Meier et al. 2016, Bourque et al. 2018; Tverin et al. 2019). The tissue biochemistry of a top predator is a proxy of its diet, and at the same time reflects the structure and biochemistry of the whole food web that can be influenced by global climate change, local human impact and natural environmental changes (Iverson et al. 2007; Engelhard et al. 2014; Lind et al. 2017). The idea of using an aquatic top predator species as a sampling device to monitor the food web is currently widely acknowledged, but the approach does not come without methodological difficulties. In this work, we studied the possibilities to improve the accuracy of marine mammal diet assessment using blubber FA profiles. For this, we used adult male grey seals *Halichoerus grypus* from the Baltic Sea, as a model. The species is widely spread on both sides of the North Atlantic, and found in several related large bays and seas, such as the Baltic Sea where grey seal is the largest and most abundant top predator. The Baltic Sea food webs, from plankton communities via fish stocks to predator populations, have in a few decades undergone large changes (Vuorinen et al.

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1998; Wasmund and Uhlig 2003; Österblom et al. 2007; Casini et al. 2008; Kauhala et al. 2017; Lind et al. 2017). Creating reliable methods to monitor spatial and temporal changes in the diets of the seals may offer means to obtain improved understanding of the predator–prey dynamics as well as early warnings of future development in the Baltic Sea and, furthermore, the methods are equally applicable to ocean ecosystems globally.

The method used in the current study is based on the principle that prey tissue FAs are incorporated into predator tissues in a predictable way although the FA compositions of a predator and its prey are not exactly the same (e.g. Budge et al. 2004; Grahl-Nielsen et al. 2011; Guerrero and Rogers 2017). Metabolic modifications of FA structures are processes that start at the absorption phase and continue after the selective incorporation into different tissue lipid as ceaseless tissue lipid turnover and remodelling (Cooper et al. 2005). In dietary studies, these issues have largely been avoided by employing calibration factors (Iverson et al. 2004; Cooper et al. 2005; Bromaghin et al. 2016). However, marine mammal blubber is not homogeneous but functionally a complex tissue, which creates a challenge for dietary monitoring. The blubber has a dual role as an energy store and as a thermal insulator (Rosen et al. 2007; Liwanag et al. 2012). The outermost layer of blubber is subjected to the largest temperature variations, directly measured with phocids in the classic work of Irving and Hart (1957). In line, several studies have reported a unique FA composition for the phocid outermost blubber (e.g. Wheatley et al. 2007; Strandberg et al. 2008, 2011; Guerrero et al. 2016; Guerrero and Rogers 2017). Such vertical FA stratification seems to be a general trait of marine mammal blubber. Outer blubber usually contains higher relative concentrations of 14–18 carbon monounsaturated FAs (MUFAs) and the inner layers are enriched with saturated FAs (SFAs), 20–22 carbon MUFAs and polyunsaturated FAs (PUFAs) (e.g. Käkelä and Hyvärinen 1996; Strandberg et al. 2008; Waugh et al. 2014; Guerrero et al. 2016; Meier et al. 2016; Guerrero and Rogers 2017; Bourque et al. 2018). As can be learned from poikilotherms, sufficiently low melting points for animal tissue in cold water can be obtained either by enriching the tissue lipids with PUFAs or MUFAs (Radnaeva et al. 2017). Thus, the reasons for the preference of MUFAs over PUFAs in the outermost blubber layer need to be considered based on the tissue requirements of mechanical strength or differently adjusted metabolic dynamics between the covering insulating and the inner metabolically active layers (Budge et al. 2004; Rosen et al. 2007, Liwanag et al. 2012; Louis et al. 2015). The layers beneath the outer blubber layer, i.e. the middle and inner blubber layers, are thermoneutral (Irving and Hart 1957), and thus the middle blubber integrates dietary FAs without restrictions due to FA melting points or variations in tissue temperature. Thereby, the middle blubber has a better capacity than the outer blubber to incorporate all types of dietary FAs, and the innermost layer closest to muscle is likely the metabolic interphase where recent dietary deposition or mobilization of all the dietary FA types occurs most actively (Budge et al. 2004; Strandberg et al. 2008; Guerrero et al. 2016; Bourque et al. 2018).

In a dietary study, it is essential to choose the blubber sample that best represents the diet of the time period of interest. Although the stratification of blubber FAs in marine mammals is firmly established and confirmed in several studies, many cited above, the question of whether an averaged whole-depth blubber sample serves as a reliable and sensitive proxy of diet, or whether small subsamples from the thermoneutral middle or inner blubber layers should be used for dietary studies, still prevails. One could also consider how sensitive the results of the dietary studies are for these choices of the sample. A further challenge to the question is the published detailed vertical FA profiles from skin to muscle with 3-mm sampling intervals (Strandberg et al. 2008, 2011), which showed that in ringed seals *Phoca hispida*, the magnitude of vertical stratification varies significantly on an individual level. These studies also showed that vertical stratification, when it occurs, is very strong for some FAs but less prominent for others. Furthermore, in many individuals, the FA gradients from skin to muscle were not linear but three separate layers, outer, middle and inner blubber, could be distinguished (Strandberg et al. 2008).

In this grey seal work, we at first confirmed the occurrence of vertical compositional differences between the outer and middle blubber of adult males, and studied the degree of individual variability in FA composition at different blubber depths. After these confirmatory examinations, we addressed our first main question not yet studied systematically: which factors drive the vertical FA stratification separating the outer blubber from middle blubber, observed in some individuals but not in others? As the possible inducers of stratification, we considered (a) Δ9-desaturation of SFAs to MUFAs in blubber, (b) different dietary FA supply, (c) age of the animal, and (d) blubber total depth. In the case of bycaught or shot wild specimens, the dietary FA supply cannot be recorded. In this study, the plausible dietary individual differences were inferred from the collection area and the fish species targeted by the trap types that bycaught or attracted these seal individuals (Table 1). Since published research of a variety of seals and whales consistently regards dietary influence on inner blubber FA composition to be strong (e.g. Koopman et al. 1996; Skoglund et al. 2010, Strandberg et al. 2011; Guerrero and Rogers 2017; Bourque et al. 2018; Tverin et al. 2019), the inner blubber FAs were used as a possible explanatory factor for the outer blubber becoming distinct from the underlying thermoneutral blubber. The implicit prerequisite for such vertical FA stratification would be that the outer layer, with its endogenously

Table 1 Background information on all the studied adult male grey seal individuals ($n = 30$) included in the study and placed in groups by PCA Euclidian dendrograms

ID	Age (years)	Blubber (mm)	ICES area	Collection method	Target fish species	Date	T °C/Δ9-DI
Group T							
5083	13	42	30	Found in trawl	Herring	25.11.2012	4.4–4.5/5.96
1613	28	42	30	Found in trawl	Herring	28.11.2012	4.0–4.2/3.35
1610	10	60	30	Found in trawl	Herring	10.11.2012	4.1–4.4/4.72
1629	19	45	30	found in trawl	Herring	08.11.2012	4.3–4.7/4.63
Group B							
1574	17	36	32	Found in bottom fyke[a]	Perch, pikeperch, cyprinids	13.08.2012	16.8–17.7/5.44
1616	13	39	29	Shot in net	–	13.11.2012	12.2–13.3/5.20
1618	13	54	29	Shot in net	–	13.11.2012	12.2–13.3/5.30
1598	11	42	29	Found in bottom fyke	Pikeperch	11.10.2012	12.6–13.2/4.27
1596	9	45	32	Found in bottom fyke	Salmon, trout, common whitefish, perch, pikeperch	11.10.2012	11.9–12.3/6.71
1615	22	36	29	Shot close to net	Pikeperch	22.11.2012	11.7–12.1/4.04
1624	10	42	32	Found in bottom fyke	Perch, pikeperch, cyprinids	24.11.2012	7.8–8.0/4.93
1594	5	42	30	Found in bottom fyke	Perch	03.10.2012	8.8–9.1/6.03
Group G							
5953	9	42	27	Shot in open water	–	21.04.2012	3.6–3.9/4.42
5459	14	63	27	Shot in open water	–	23.11.2011	7.0–7.2/6.49
5640	6	36	27	Found in bottom fyke	European eel	21.08.2012	17.5–18.1/5.73
5902	6	45	30	Shot in open water area	–	02.11.2011	7.4 –7.4/6.29
1611	22	36	29	Shot in surface fyke[b] near fishfarm	Common whitefish	22.11.2012	11.7–12.1/4.27
5955	16	51	30	Shot in open water	–	10.11.2011	7.2 –7.4/4.36
1617	13	36	29	Shot close to net	Pikeperch	13.11.2012	12.2–13.3/5.61
1619	13	36	29	Shot close to net	Pikeperch	13.11.2012	12.2–13.3/5.22
5765	6	39	27	Found in bottom fyke	–	27.09.2012	12.2–12.4/4.72
5760	5	39	27	Found in bottom fyke	–	24.09.2012	13.0–13.4/5.25
5794	11	36	27	Found in bottom fyke	–	10.10.2012	11.3–11.4/4.97
1625	14	90	30	Shot near fishfarm	–	30.12.2012	1.3–1.4/4.81
Remaining							
5922	9	36	29	Shot in open water area	–	17.04.2010	NA[c]/5.04
1620	13	48	29	Shot close to net	Pikeperch	13.11.2012	12.2–13.3/5.56
5643	6	36	30	Shot close to net	–	29.10.2011	7.5–7.6/4.37
5908	25	45	27	Shot in open water	–	21.04.2012	3.6–3.9/4.17
1595	34	57	32	Found in bottom fyke	Cyprinids	17.10.2012	11.6–11.7/4.73
1606	7	48	32	Found in surface fyke	Salmonids, common whitefish	07.11.2012	8.8–9.2/6.35

The age, blubber depth, ICES area (ESM 1) where the seal individual was collected from, as well as gear type and target fish species attempted to catch with the gear are listed. In addition, the collection date and temperature of surface water of the day (min − max) from the nearest monitoring buoy and the Δ9-DI of the outer blubber of the individual are presented

[a]Bottom fyke = fyke of various traditional structures, placed at the bottom, and was meant to catch perch, pikeperch and cyprinids

[b]Surface fyke = fyke with a floating push-up design and was meant to catch large pelagic species, e.g. salmon, trout and common white fish

[c]No information, next available measurement 6.0 °C on 25.5.2010

high degree of FA Δ9-desaturation, does not reflect diet in the same degree as the dietary FA-receiving inner blubber, and consequently the vertical differences in Δ9-desaturation were also considered as a stratification-inducing factor.

The crucial question for pinniped diet monitoring programmes is whether the neglect of vertical stratification of blubber FAs compromises the studies, i.e. does examining the FA composition of the whole vertical layer of blubber, or alternatively choosing subsamples from different vertical layers give parallel or significantly different results. Could we group seal individuals into different feeding ecology groups depending on which blubber sampling practice was

used? This study with a pinniped species provides a detailed systematic analysis of the factors affecting the FA profiles in small subsamples from specific blubber depths or alternatively in samples covering the most part or the full vertical layer of the blubber, and the results advise in choosing optimal sampling protocols and reveal the benefits and limitations of these different choices.

Materials and methods

Blubber samples

Blubber samples from adult male grey seals ($n = 30$, Table 1) were collected during 2010–2012 from the Baltic Sea from International Council for the Exploration of the Sea (ICES) subdivisions (SD) 27, 29, 30 and 32 (ESM 1). The samples were collected in collaboration with ongoing national and international seal monitoring programmes, which in Sweden were promoted by the Environmental Protection Agency (www.swedishepa.se) and the Agency for Marine and Water Management (www.havochvatten.se) and conducted by the University of Agricultural Sciences (www.slu.se) and Museum of Natural History (www.nrm.se). In Finland, the samples were collected by the Natural Resources Institute (www.luke.fi). Seal sex was recorded, the age was determined based on the number of cementum zones in canine teeth longitudinal sections (Hewer 1964), and the sampling location, date, surface water temperature at nearest buoy (Finnish Meteorological Institute and Swedish Meteorological and Hydrological Institute open data; Fig. ESM1) and cause of death were recorded (Table 1). The seals were found bycaught in different gears (trawl, net, surface or bottom fyke), or shot either close to fishing gear, fish farm or in other areas (detailed with the target fish of the trap types in Table 1).

For this study, we standardized the sampled cohort by including adult individuals (≥ 5 years) with blubber thickness over 36 mm, because previous ringed seal studies (Strandberg et al. 2008, 2011) and also the current grey seal data showed that the middle layer cannot be distinguished in seals leaner than approximately 36 mm since this layer expands and shrinks due to the nutritional status of the animals. Due to the large effects of potential lactating periods on blubber dynamics and composition, the females (originally fewer individuals collected than males) were omitted from this study. The final selection of individuals allowed to specifically address the effect of blubber depth (as a proxy of nutritional status) and inner blubber FA composition (as an indirect proxy of dietary items, supported by the grouping of individual from the same location and trap types in multivariate analyses according to the blubber FAs), without potential differences in lipid mobilization from the innermost blubber hampering the study (Raclot 2003).

The collected full-depth blocks of blubber were stored at − 20 °C until analysis. FA methyl esters were prepared and analysed from subsamples of the blubber blocks (dissected with skin and muscle) according to the published protocols (Strandberg et al. 2008). Upon sampling, the blubber was frozen in liquid nitrogen, and vertical subsamples were taken at 3-mm intervals from skin to muscle (Fig. 1). The outer layer was represented by the second subsample, i.e. sampled 3–6 mm from the skin, and the inner layer was represented by the subsample from 3 to 6 mm above muscle. The

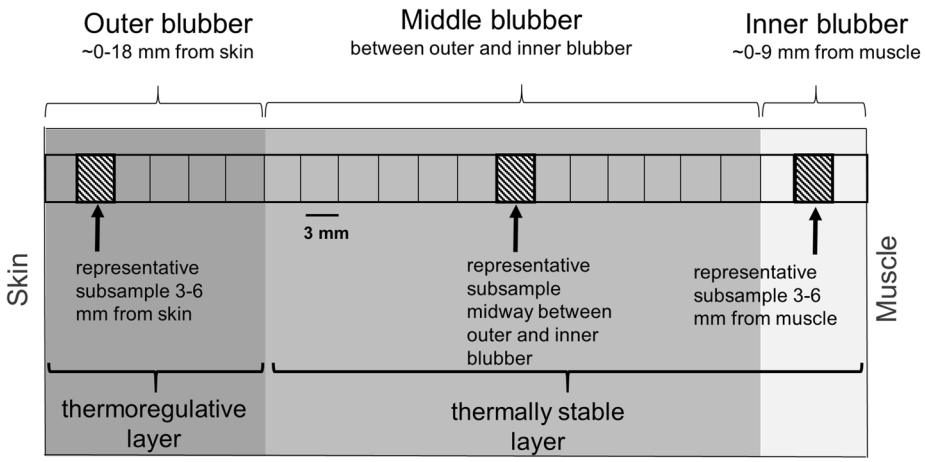


Fig. 1 Scheme of the blubber column from skin (left) to muscle (right), showing the sampling sites. Outer and inner blubber is represented by the second outermost and second innermost subsample, respectively. The subsample for middle blubber was the one nearest to the centre between the boundaries of the outer (≈0–18 mm from skin) and inner blubber layers (≈0–6 mm from muscle). Thermoregulatory and thermally stable layers of blubber are indicated. For complete vertical profiles, the blubber column was divided into subsamples 3 mm apart, which all were studied for fatty acid composition. See Fig. 2 for the justification of the three layers

outermost 3-mm and the innermost 3-mm subsamples of blubber were not used as representative subsamples due to the potential influence of skin connective tissue or muscle fibres on the fatty acid profile, respectively. As an exception, the 0–3-mm sample was used when calculating the outer blubber Δ9-desaturation index (Δ9-DI) to examine the effects of seal age on outer blubber characteristics, which ensured full comparability with previously published ringed seal data utilizing this subsample (Käkelä and Hyvärinen 1996; Strandberg et al. 2008). This study also employed the same individual MUFAs and SFAs for the calculation of Δ9-DI as were used in these previous studies. The middle layer was represented by the subsample taken equally far from the depth of 18 mm below skin (the outermost 18 mm is affected by tissue temperature changes reflecting ambient temperature) and the depth of 6 mm above muscle (the innermost 6 mm largely vary in composition and is regarded as the metabolic interphase) (Fig. 1). In this study, these boundaries of the middle layer on both sides were confirmed by studying the rate of vertical change of FA composition (numerical gradients, NGs) in the blubber column of the adult males. These boundaries were further supported by similar patterns reported previously for ringed seals (Strandberg et al. 2008, 2011).

Fatty acid analysis

The blubber FA composition was studied by a Shimadzu GC-2010 Plus gas chromatograph after conversion of the acyl chains of blubber lipids to methyl ester derivatives by heating under nitrogen atmosphere in 1% methanolic sulphuric acid (Christie 1993). With this equipment, the FA methyl esters (FAMEs) were quantified using a flame-ionization detector (FID), the response of which has a large linear range. Additionally, the FAMEs were identified using electron impact mass spectra recorded by Shimadzu GCMS-QP2010 Ultra with a mass selective detector (MSD). Both equipment were manufactured by Shimadzu Scientific Instruments (Kyoto, Japan) and equipped with Zebron ZB-wax capillary columns (30 m, 0.25 mm ID and film thickness 0.25 µm) from Phenomenex (Torrance CA, USA). The procedures of the FA analysis have been described in detail before (Käkelä et al. 2005; Strandberg et al. 2008). In short, the FAMEs were injected into the GC column by AOC-20i autoinjector at 250 °C, chromatographed using an oven temperature programme: initially at 180 °C for 8 min, raised 3 °C/min–210 °C, which was kept for 50 min. The FID was at 280 °C. The FA compositions were expressed as mol% profiles, and the FAs were abbreviated: [carbon number]:[number of double bonds]n-[position of the first double bond calculated from the methyl end] (e.g. 20:5n-3).

The FAs chosen for detailed analyses of FA vertical stratification included those 9 components (14:0, 16:1n-7, 18:1n-9, 18:2n-6, 18:3n-3, 18:4n-3, 20:1n-7, 20:4n-6, 22:6n-3) that in our previous grey seal dietary study (Tverin et al. 2019) explained most of the interspecific variation among 11 key prey fish species caught in the study area (herring *Clupea harengus;* largely subspecies *membras*, sprat *Sprattus sprattus*, Atlantic salmon *Salmo salar*, sea trout *Salmo trutta*, pikeperch *Sander lucioperca*, pike *Esox lucius*, perch *Perca fluviatilis*, common whitefish *Coregonus lavaretus*, eelpout *Zoarces viviparous*, roach *Rutilus rutilus,* European eel *Anguilla anguilla)* and thus were potential dietary markers (Lundström et al. 2010; Tverin et al. 2019). In addition, important precursors or derivatives of those dietary markers (14:1n-5, 16:0, 18:0, 18:1n-7, 20:5n-3, 22:5n-6) were included. This selection of 15 FAs included the ones potentially reflecting the diet of the seals, as well as the ones that are quantitatively most important.

Determination of distinct vertical blubber layers and comparison of their fatty acid composition

Complete vertical profiles of FAs in the blubber column of adult grey seal males were constructed by calculating the mol% of each studied FA in every subsample across the blubber column. The presence of biochemically distinct vertical layers was confirmed by studying the rate of change in FA composition for each blubber depth. To demonstrate the vertical compositional change, the five quantitatively most significant FAs (16:1n-7, 18:1n-9, 16:0, 20:5n-3 and 22:6n-3) were studied for the change in their percentage value against the previous and next sample in the sequence, and the differences as absolute values, obtained on both sides, were averaged. Subsequently, these absolute and averaged mol% differences for each FA at each point of blubber depth were summed to form a numerical gradient (NG) representing all the studied FAs at this depth. Finally, the NG was plotted as a function of blubber depth. The "outer" and "inner" layers could be distinguished from the NG curves as areas having NG values significantly higher than the lowest reference value of the "middle" blubber. The NGs were compared using paired Student's *t* test, with $P < 0.05$ as significance level. The first significantly different NG value towards to skin or muscle sets the boundary against the middle layer.

To test for differences in the relative concentrations of different FAs between the distinguished blubber layers, box–whisker diagrams were compiled using the FA data from representative subsamples of the different layers, and the univariate comparisons between the FA percentages in the different layers were performed by non-parametric ANOVA employing Šidák correction for multiple comparisons (IBM SPSS version 24.0, New York, USA). To address FA variability at different blubber depths, the equality of variances of the layers were investigated by *F* tests. In these tests, $P < 0.05$ was used as the level of statistical

significance. The Δ9-DI was calculated according to Käkelä and Hyvärinen (1996), and used as one potential factor driving stratification.

Stratification index of blubber fatty acids

To assess vertical distribution tendency for each FA along the depth of the blubber, we calculated a Stratification Index (SI, Eq. 1, modified from Olsen and Grahl-Nielsen 2003) using the mol% values of the studied FAs in the representative subsamples of the outer and middle blubber layers. Dividing the differential of outer and middle layer mol% with the mean of these yielded a numerical index which is positive if the mol% of a certain FA is higher in the outer layer than it is in the middle layer. The mol% values from the inner blubber were not incorporated into the equation since (a) in the subsequent analyses, the index was plotted as a function of the inner layer mol% values, and (b) the largest vertical compositional change was found between the outer and inner blubber, as indicated by the NG curves.

$$\mathrm{SI} = \frac{\mathrm{mol\%_{(outer)}} - \mathrm{mol\%_{(middle)}}}{\mathrm{mol\%_{(outer)}} + \mathrm{mol\%_{(middle)}}/2}. \quad (1)$$

We performed two types of statistical tests. First, we calculated the SI for Δ9-DI using the middle and outer blubber layer values. The SI of Δ9-DI was then used as dependent variable in a factorial generalized linear model (GLM) with Δ9-DI of the inner blubber, seal age and blubber thickness as explanatory variables. In this model, blubber thickness was not significant in any interaction or as main factor (data not shown) and, therefore, was not included in subsequent tests. Blubber thickness was also removed since it on purpose had been pre-selected to be within a certain range. The second set of tests used the same model framework as above (factorial GLM) but had SI of individual FAs as dependent variable and FA mol% in inner blubber and age as explanatory variables. The interaction between age and inner blubber layer FA mol% was removed if non-significant. These GLM tests were performed using IBM SPSS version 24.0 (New York, USA). The model parameters, blubber SI versus inner blubber FA mol%, were also used to determine the threshold FA mol% values of inner blubber that in the grey seals necessitate vertical FA stratification from the middle to outer blubber (details in Fig. 9).

Multivariate PCA to indicate differences in fatty acid composition

Multivariate analyses (principal component analysis, PCA) (Sirius 8.5 software, Pattern Recognition Systems, Bergen, Norway) were conducted to assess compositional differences between the sample layers and to highlight the blubber layer in which the largest intra- and inter-individual variation of the data are found. Prior to the analysis, FA data were arcsine transformed and all FA variables standardized to prevent large components from dominating the analysis (when employing covariance matrix). The relative positions of the samples and variables were plotted using the first two principal components, and separations between sample groups were tested for statistical significance using Soft Independent Modeling of Class Analogy (SIMCA, Wold and Sjöström 1977, Sirius 8.5) with $P < 0.05$ as significance level. For the comparisons of the different procedures of taking blubber samples (subsamples from different depth or averaging FA profiles over full depth or certain vertical layers), seal groups were formed according to PCA utilizing Euclidian distances of inner blubber FA composition of the seals (Table 1).

Results

Vertical biochemical layers of grey seal blubber

To investigate the magnitude of change of FA composition at different depths of the blubber, the sum of the NGs for the quantitatively most important FAs were plotted as a function of blubber depth (Fig. 2). The largest NG values in the FA composition were observed in the outer blubber layer, as indicated by statistically significantly elevated values compared to the lowest (reference) value found in a subsample of the middle layer ($P < 0.05$, Student's t test). To depict these changes accurately and to avoid discrepancies due to varying blubber depth (36–90 mm) of the studied grey seals, individuals ($n = 10$) with a blubber depth of 36 mm were plotted in panel a, individuals ($n = 14$) with a blubber depth of 39–48 mm in panel b, and individuals ($n = 4$) with the blubber thickness of 54–63 mm in panel c. In all the three ranges of blubber depth, the NGs of the 5–7 most superficial subsamples significantly differed from the reference point. In the individuals with blubber thickness of 39–48 mm, also the innermost subsamples at the depth of 39–45 mm differed significantly from the reference point. Next, bidirectional vertical plot of NGs (starting from the outermost or the innermost layer, irrespective of the total blubber thickness) was drawn for all the individuals ($n = 30$), and the graphs confirmed the presence of the distinct outer and inner layers with transition depths at 15–18 mm from skin and 9 mm from muscle, respectively (Fig. 3).

Saturated and monounsaturated fatty acids stratify extensively: polyunsaturated fatty acids less

The relative concentrations of the studied FAs (mol%) in the representative subsamples of the outer, middle and inner layers of blubber from the adult grey seals were

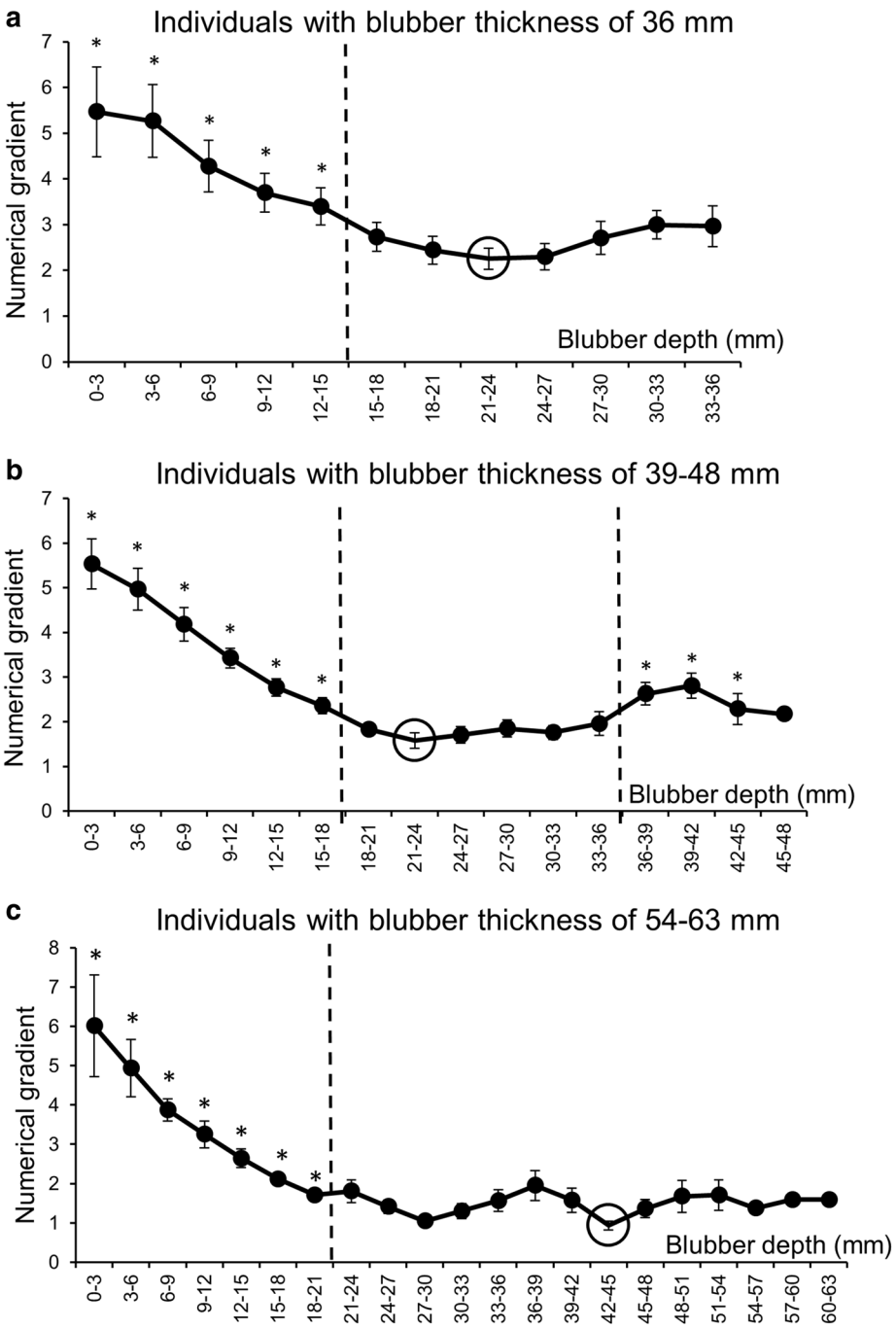


Fig. 2 Vertical profiles of numerical gradients (NGs) from the main fatty acids (FAs) of adult grey male seals. Numerical gradients of five main FAs (16:1n-7, 18:1n-9, 16:0, 20:5n-3 and 22:6n-3) in the blubber column of adult grey male seals having a blubber thickness of **a** 36 mm ($n=10$), **b** 39–48 mm ($n=14$) and **c** 54–63 mm ($n=5$) are presented. The reference subsamples for statistics are marked with a large open circle (in panels **a** and **b**, the subsample 8 at 21–24 mm depth, and in panel c the subsample 15 at 42–45 mm), and the subsamples where the numerical gradient significantly ($P<0.05$, Student's *t* test) differs from that of the reference point (blank circle) is marked with an asterisk. Depths with the incipient compositional change (i.e. boundary sites for outer–middle and middle–inner blubber layers) are marked with dashed lines. For easy scaling, one individual with exceptional blubber depth of 90 mm was omitted from panel **c** but was included in Fig. 3. Error bars represent ± standard error

plotted as box–whisker diagrams (Figs. 4, 5, 6). The outer blubber percentages for 14:0, 16:0, 18:0, 16:1n-7, 20:1n-7, 18:4n-3, 20:4n-6, 20:5n-3 and 22:6n-3 showed significantly less individual variation than those of the middle or inner layers (seen as unequal variation in the *F* test, $P<0.05$, and smaller difference between max and min values). No statistically significant difference in the magnitude of the individual variation for any of the FA mol% was found between the middle and inner blubber.

The outer blubber layer consistently contained significantly smaller relative concentrations of all individual SFAs (Fig. 4) and larger concentrations of MUFAs 14:1n-5, 16:1n-7 and 18:1n-9 (the Δ9-desaturated ones with no further elongation) (Fig. 5) than the middle and inner layers (all $P<0.05$). For the main SFAs, and the MUFAs 14:1n-5 and 16:1n-7, also the middle and inner layers had significantly different relative concentrations. Regarding PUFAs (Fig. 6), the outer layer composition differed significantly from that

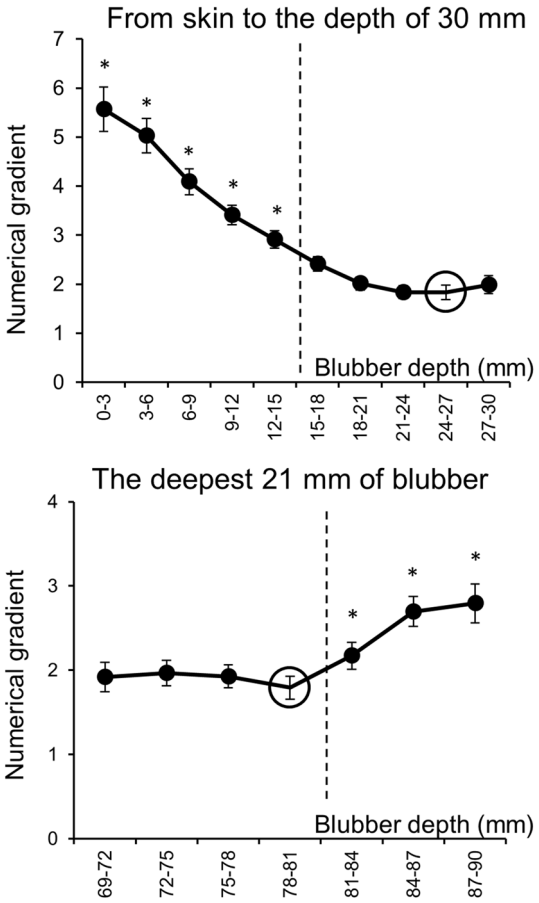


Fig. 3 Bidirectional vertical profiles of numerical gradients (NGs) from the main fatty acids in the blubber column of all the studied adult grey male seals ($n=30$). The NGs starting from the outermost blubber layer (NG shown from the skin to the depth of 30 mm) or the innermost layer (NG shown from the deepest 21 mm of the blubber column), irrespective of the total blubber thickness are presented. Reference subsamples are marked with a large open circle, and the depth where the numerical gradient significantly ($P<0.05$, Student's t test) differs from that of the reference point is marked with an asterisk. Error bars represent ± standard error

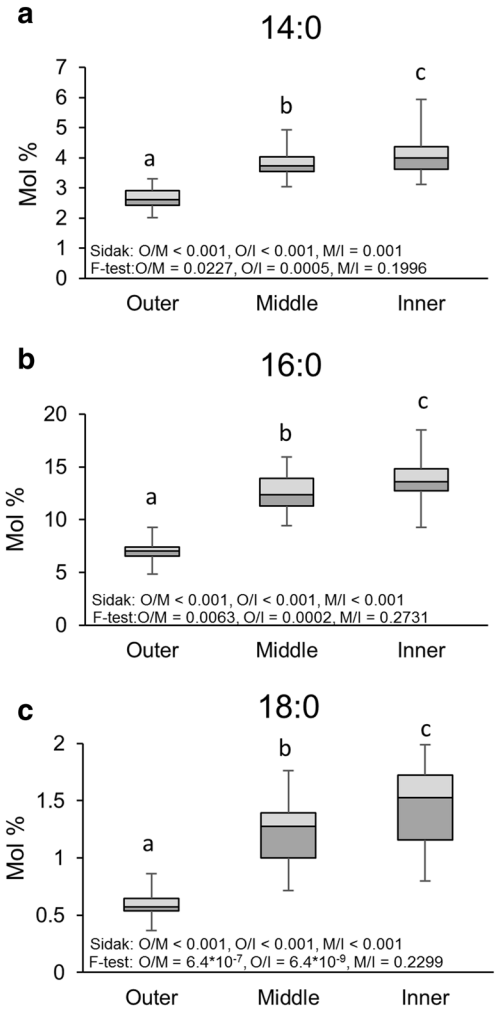


Fig. 4 Box–whisker diagrams of the main studied saturated fatty acids in the blubber of adult male grey seals ($n=30$). **a** 14:0, **b** 16:0 and **c** 18:0 in the outer, middle and inner blubber of adult male grey seals are presented. The boxes show the median and upper and lower quartiles, and the whiskers indicate the highest and lowest value of the data. Above the diagrams, different letters indicate significantly different fatty acid percentages in the different layers ($P<0.05$, non-parametric ANOVA employing Šidák correction for multiple comparisons). Results from F tests ($P<0.05$) investigating equality of variances in the different blubber layers (O outer, M middle, I inner) are inserted as text

of the middle and inner layers by having more 18:2n-6 and 18:3n-3, and less 18:4n-3, 20:4n-6 and 20:5n-3. The individual PUFA percentages of the middle and inner layers were the same, except 20:5n-3 was slightly more abundant in middle layer.

When the adult male grey seal ($n=30$) blubber FA data containing the representative subsamples from every vertical layer (outer, inner and middle) were subjected to multivariate PCA, and the statistical significance of the compositional difference was tested by SIMCA, the outer blubber composition revealed to be significantly different from that of the middle and inner layers. The outer layer was enriched in MUFAs such as 14:1n-5, 16:1n-7, and 18:1n-9, whereas the middle and inner layers were especially rich in SFAs such as 14:0, 16:0 and 18:0 (Fig. 7).

Since the vertical differences in the levels of SFAs and MUFAs were large, special attention was paid to the FA Δ9-desaturation (converting SFAs to MUFAs) as a factor creating vertical FA stratification. Thus, we run a GLM using SI for Δ9-DI (outer versus middle blubber) as a dependent variable, and the Δ9-DI of inner blubber, seal age and its blubber thickness as the explanatory variables. In this test (Table 2), the Δ9-DI of inner blubber and seal age were significant effects but blubber thickness (pre-selected to be within a certain range) was not, even as a main effect and, therefore, was excluded from further modelling of the vertical

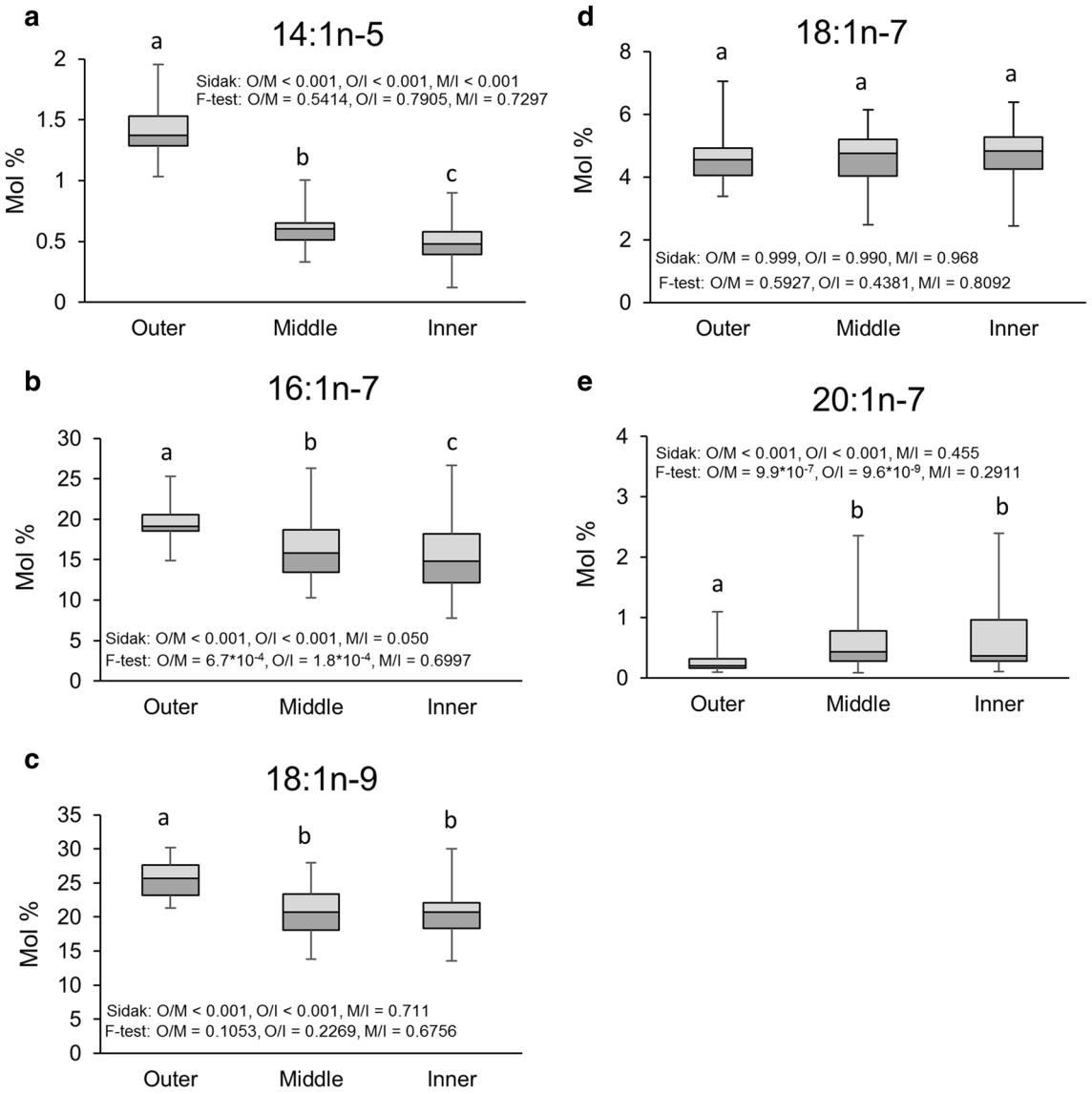
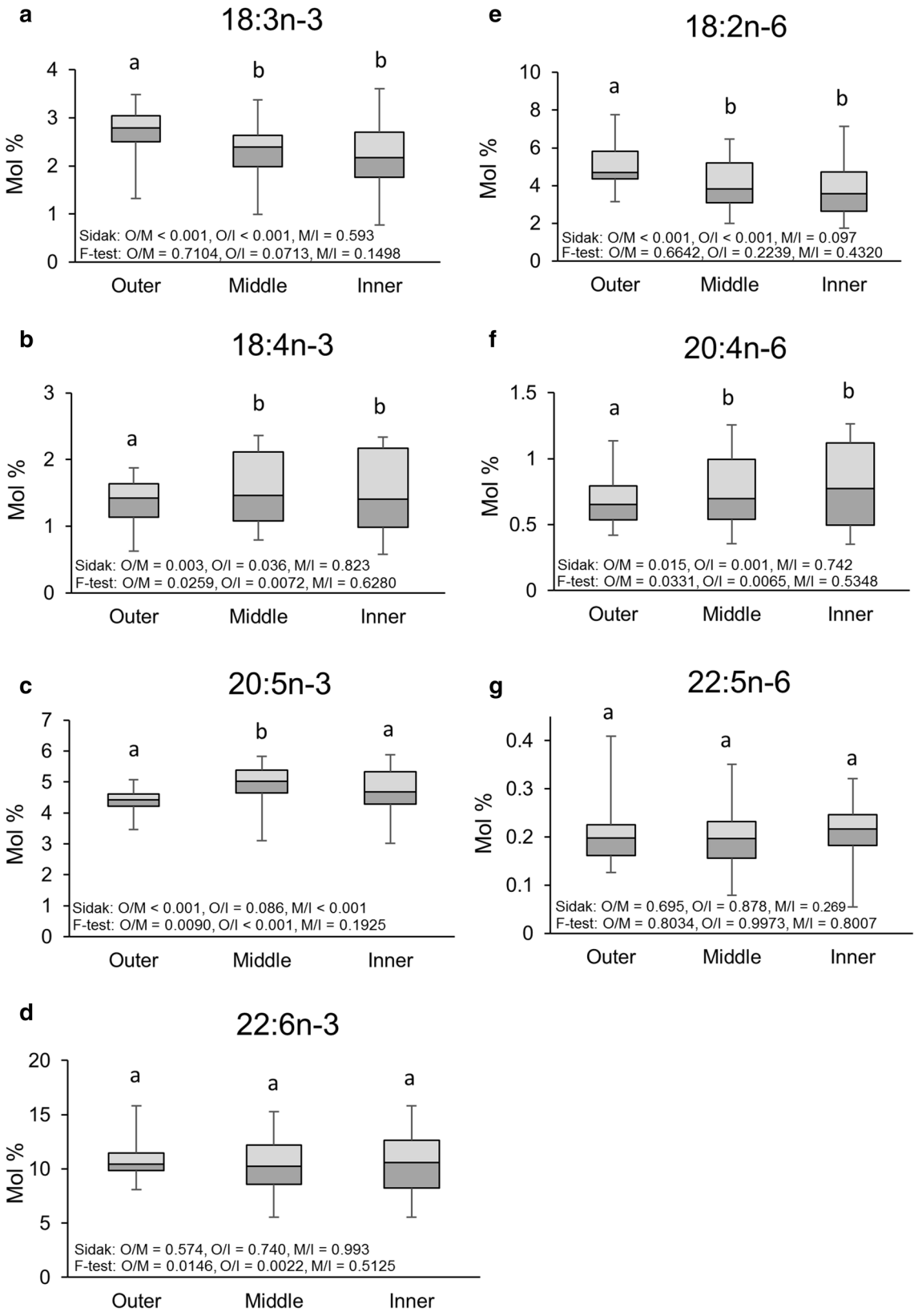


Fig. 5 Box–whisker diagrams of the main studied monounsaturated fatty acids in the blubber of adult male grey seals ($n=30$). **a** 14:1n-5, **b** 16:1n-7, **c** 18:1n-9, **d** 18:1n-7 and **e** 20:1n-7 in the outer, middle and inner blubber of adult male grey seals are presented. The boxes show the median and upper and lower quartiles, and the whiskers indicate the highest and lowest values of the data. Above the diagrams, different letters indicate significant difference in fatty acid percentages in the different layers ($P<0.05$, non-parametric ANOVA employing Šidák correction for multiple comparisons). Results from F tests ($P<0.05$) investigating equality of variances in the different blubber layers (*O* outer, *M* middle, *I* inner) are inserted as text

distribution of individual FAs. The outer blubber Δ9DI was found to correlate negatively with the age of the adult grey seal males of this study, while the outer blubber PUFA contents were not affected by age (Fig. 8). Moreover, the season and the water temperatures measured in the surface water during the days when the grey seals of our study were collected (3.6−18.1 °C) had no significant effect on their outer blubber Δ9-DI (Table 1). The SI of Δ9-DI (inner versus middle blubber) got positive values for all the studied individuals with any detected inner blubber Δ9-DI values (Fig. 9a), meaning that in all the individuals, the overall degree of Δ9-desaturation was consistently increased towards the skin. In addition, those individuals that had relatively low Δ9-DI of inner blubber showed the largest increase of Δ9-DI from middle to outer blubber.

Degree of vertical fatty acid stratification is determined by varying fatty acids of the innermost blubber contrasting uniform fatty acids of outer blubber

Since SI for Δ9-DI was only determined by alterations in SFAs and MUFAs metabolically linked to FA


a
18:3n-3
Mol %
a
b
b
Sidak: O/M < 0.001, O/I < 0.001, M/I = 0.593
F-test: O/M = 0.7104, O/I = 0.0713, M/I = 0.1498
Outer
Middle
Inner
e
18:2n-6
Mol %
a
b
b
Sidak: O/M < 0.001, O/I < 0.001, M/I = 0.097
F-test: O/M = 0.6642, O/I = 0.2239, M/I = 0.4320
Outer
Middle
Inner
b
18:4n-3
Mol %
a
b
b
Sidak: O/M = 0.003, O/I = 0.036, M/I = 0.823
F-test: O/M = 0.0259, O/I = 0.0072, M/I = 0.6280
Outer
Middle
Inner
f
20:4n-6
Mol %
a
b
b
Sidak: O/M = 0.015, O/I = 0.001, M/I = 0.742
F-test: O/M = 0.0331, O/I = 0.0065, M/I = 0.5348
Outer
Middle
Inner
c
20:5n-3
Mol %
a
b
a
Sidak: O/M < 0.001, O/I = 0.086, M/I < 0.001
F-test: O/M = 0.0090, O/I < 0.001, M/I = 0.1925
Outer
Middle
Inner
g
22:5n-6
Mol %
a
a
a
Sidak: O/M = 0.695, O/I = 0.878, M/I = 0.269
F-test: O/M = 0.8034, O/I = 0.9973, M/I = 0.8007
Outer
Middle
Inner
d
22:6n-3
Mol %
a
a
a
Sidak: O/M = 0.574, O/I = 0.740, M/I = 0.993
F-test: O/M = 0.0146, O/I = 0.0022, M/I = 0.5125
Outer
Middle
Inner

◂**Fig. 6** Box–Whisker diagrams of the main studied polyunsaturated fatty acids in the blubber of adult male grey seals ($n = 30$). **a** 18:3n-3, **b** 18:4n-3, **c** 20:5n-3, **d** 22:6n-3, **e** 18:2n-6, **f** 20:4n-6, and **g** 22:5n-6 in the outer, middle and inner blubber of adult male grey seals are presented. The boxes show the median and upper and lower quartiles, and the whiskers indicate the highest and lowest values of the data. Above the diagrams, different letters indicate significant difference in the fatty acid percentages in the different layers ($P < 0.05$, non-parametric ANOVA employing Šidák correction for multiple comparisons). Results from F tests ($P < 0.05$) investigating equality of variances in the different blubber layers (O outer, M middle, I inner) are inserted as text

Δ9-desaturase, we ran separate models for SI of structurally diverse FAs, where the FA SI (outer versus middle blubber) was the dependent variable and the FA mol% of inner blubber and age were explanatory variables (Table 3). In most cases, only FA mol% of inner blubber had a statistically significant effect on the SI of that FA. Age only affected the SI for 16:0 and 16:1n-7 with the slight correlations being positive and negative, respectively. In only one case, SI of 16:1n-7 was the combined effect (slight positive correlation with inner blubber FA proportion × seal age) significant. These data clearly indicated that the FA composition of the innermost blubber layer, which varies between the individuals while the outer blubber is uniform, influences the vertical FA stratification in the grey seal blubber (Table 3). The strongest dependencies with the highest R^2 values (> 0.5) between the SI and inner blubber proportion of the FA were found for 16:1n-7, 18:1n-9 and 22:6n-3 (Fig. 9). Examples of the full vertical profiles of the five quantitatively most important FAs were presented for four individuals having either high or weak FA stratification (Fig. ESM2).

The inner layer subsample indicates the blubber compositional variation of fatty acids the clearest

To determine the blubber layer that represents individual variation of FA composition most efficiently, at first PCA-based Euclidian dendrogram was allowed to create test clusters of the studied individuals for the PCA using the FA composition of the inner blubber subsamples (Fig. 10a). These PCA-created clusters were colour coded and further followed in subsequent PCAs using either the FA compositions of blubber subsamples from different depths or the FA compositions averaged over full blubber depth or over certain vertical layers (Fig. 10a–f). When using the middle blubber subsamples, two groups marked T (Trawl) and R (Red) were no longer separated from each other according to SIMCA (Fig. 10b). A large decline in the separation power was found when the outer blubber subsamples were used, and only two out of the original six pairwise significant SIMCA comparisons remained (Fig. 10c). To understand the effect of including the different vertical layers on the blubber FA-based grouping of the seal individuals, the PCA biplots were studied for the FA composition averaged over full depth of blubber (Fig. 10e), the averaged composition of the combined inner and middle layers (Fig. 10d, omitting the six outermost subsamples), and the mean composition of the combined middle and outer layers (Fig. 10f, omitting the three innermost subsamples). Among these samples, the sample that omitted the outer blubber subsamples showed the clearest separations (with all paired SIMCA tests statistically significant) resembling those obtained using only the one representative inner blubber subsample (Fig. 10a, d). In addition, the sample averaging the full-depth of blubber was able to separate most of the test groups of the individuals but failed to detect the difference between the T and R groups (Fig. 10e). Leaving out the innermost layer impaired the resolution more and two pairwise SIMCA comparisons showed non-significant result (Fig. 10f). Since the endogenous metabolism (e.g. Δ9-desaturation) in the outer blubber modifies the SFA and MUFA components largely, the PCA for the outer subsamples were repeated using all detected PUFAs (which all are diet derived) of the samples, which selected set of FAs was renormalized to 100 mol% to fully remove the influence of SFAs and MUFAs. Consequently, the ability of the outer blubber subsamples to separate the test groups was improved and also the R and Blue (B) groups got separated. Now three out of the six pairwise SIMCA comparisons showed statistically significant difference (Fig. 11).

Discussion

The FA composition in marine mammal blubber is regarded as a valuable source of information on the feeding ecology of the animals and at the same time as a proxy of the structure of the food web (Bradshaw et al. 2003; Thiemann et al. 2007). The reports of the very clear vertical differences in seal blubber FA composition, however, raised concern about how reliable this information is since the FA profile largely depends on the depth from where the subsample is taken (e.g. Best et al. 2003; Thiemann et al. 2004; Strandberg et al. 2008; Liwanag et al. 2012; Guerrero et al. 2016). Since the stratification process of different FAs in the blubber has not yet been understood or systematically studied, the responsible recommendations at first advised to take a sample comprised of the whole vertical blubber column from skin to muscle, and to study its FA composition (Thiemann et al. 2004; Budge et al. 2006). Since the degree of this vertical FA stratification of grey seal blubber varied individually, as was reported for the ringed seals as well (Strandberg et al. 2008, 2011), in the present study, we examined which factors drive the stratification in the blubber of a widely distributed species, the grey seal. Our data support the view that due to fairly stable FA composition of the outer blubber, the

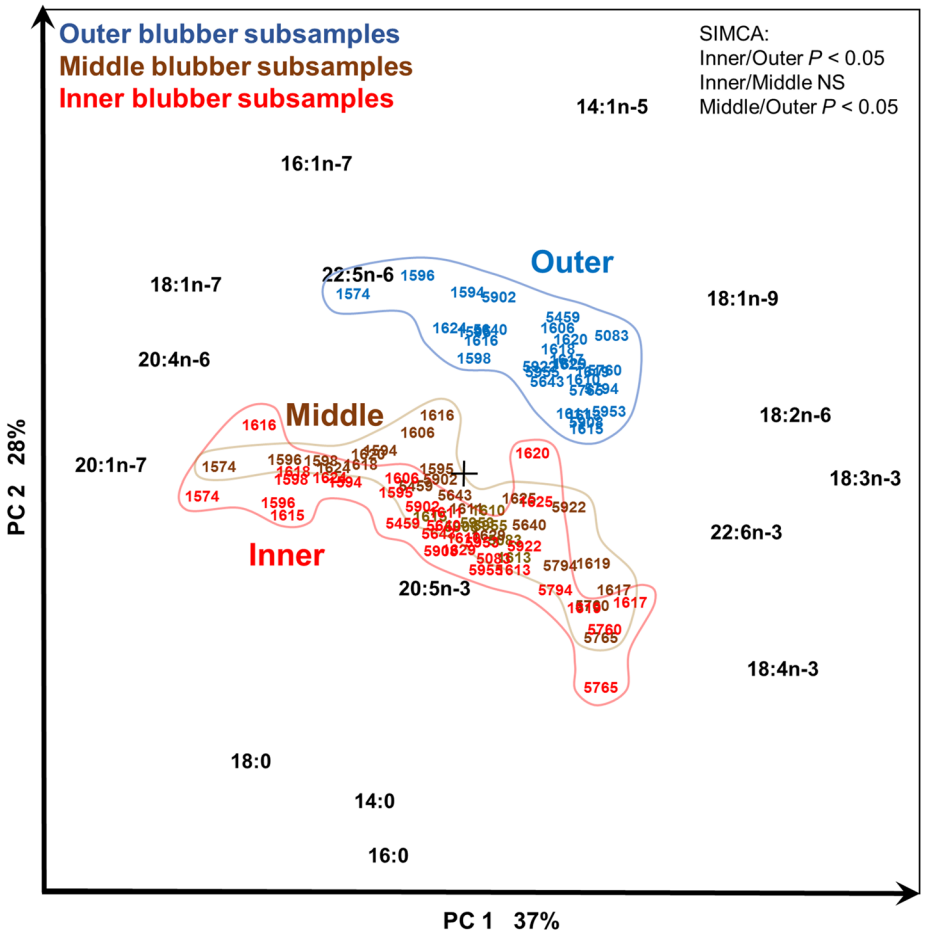


Fig. 7 PCA biplot of the fatty acid composition in outer, middle and inner blubber of the studied adult male grey seals ($n = 30$). Outer (blue), middle (brown) and inner (red) blubber samples of the adult male grey seals (marked by the identification code of the individual) are presented using the 15 main fatty acids as loadings. SIMCA tests of the statistical significance of the compositional differences ($P < 0.05$) is reported as text insert

Table 2 Generalized linear model (GLM) analysis of the effect of Δ9-DI in the inner blubber layer and the seal age on the Δ9-DI stratification index (SI, for outer versus middle blubber) in adult male grey seals ($n = 30$)

Effect	*B*	Std. Error	LWC	UWC	Wald χ^2	*df*	*P*
Intercept	1.242	0.165	0.918	1.566	56.422	1	< 0.001
D9-DI-inner	− 0.199	0.069	− 0.334	− 0.064	8.37	1	0.004
Age	− 0.008	0.004	− 0.016	< 0.001	3.956	1	0.047

B coefficient of the dependency, *LWC* lower 95% Wald confidence interval, *UWC* upper 95% Wald confidence interval, *Wald* χ^2 Wald Chi squared test value

degree of FA stratification in the blubber column, varying individually, is dependent on the individual variations in the inner blubber FA composition, originating from different diet. These data also gave us the opportunity to revisit the question of which is the most reliable method for sampling seal blubber for dietary studies.

Vertical biochemical layers of grey seal blubber

The phenomenon of gradual vertical stratification of blubber FAs is widely accepted in pinnipeds (e.g. Käkelä and Hyvärinen 1996; Wheatley et al. 2007; Fowler et al. 2014; Guerrero et al. 2016) but the idea of blubber having separate vertical layers with different FA composition and even distinguishable transition zones was first proposed for ringed seals (Strandberg et al. 2008, 2011). The separate layers presumably arise due to the dual role of blubber as (i) a thermoregulatory and (ii) an energy storage tissue. The present data on grey seal blubber FAs confirm the presence of such biochemically divergent vertical layers but at the same time shows that the magnitude of the layering is highly variable among different individuals, even though all the individuals of this study were adult males with thick blubber. The thicknesses of the outer and inner blubber layers (identified

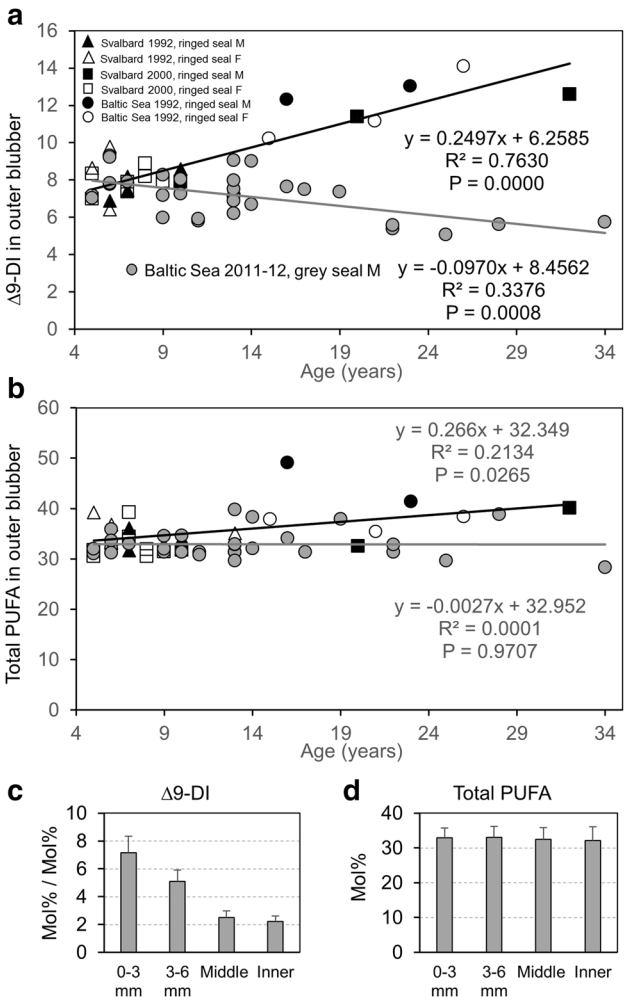


Fig. 8 Grey seal and ringed seal (**a**) desaturation index Δ9-DI and **b** total polyunsaturated fatty acid (PUFA) content of outer blubber (0–3 mm sample) as function of seal age. The grey seals of this study, collected from the Baltic Sea in 2010–2012, are compared with the ringed seals collected from Svalbard in 1992 and 2000, and in the Baltic Sea in 1992 (originally analysed for Käkelä and Hyvärinen 1996, and Strandberg et al. 2008, and used as reference with full permission from the authors). The average values for **c** Δ9-DI in different vertical layers of the grey seals reflect sampling depth but the values for **d** total PUFA were stable

based on the vertical NG curves indicating the magnitude of compositional change from one subsample to the next one, located above or beneath) of the grey seals were surprisingly similar to those reported for the ringed seals (Strandberg et al. 2008, 2011). In both species, the outer blubber characteristics fade after the depth of about 15 mm from the skin, and then the middle layer, which expands and shrinks due to the nutritional status of the animal, has stable FA composition, until the last about 9 mm of blubber adjacent to muscle, which again show a FA composition differing from the other blubber layers. In the classic work of Irving and Hart (1957), the same 15-mm layer of harbor seal outer blubber was reported to be poikilothermic, i.e. the tissue temperature reflected the ambient temperature. Beneath this depth, the blubber was thermoneutral, which suggests it serves as a normal energy depot. In two ringed seal subspecies (Arctic marine *Phoca hispida hispida* and lacustrine *Phoca hispida saimensis*), this outer blubber layer showed much compositional similarity whereas the middle and inner layers had different FA composition closer to that of the potential dietary fish (Strandberg et al. 2011). Thus, it seems likely that in marine mammals, the outer blubber FA composition is endogenously regulated and less affected by changes in dietary FA supply than the layers beneath the 15 mm from skin. The deepest layer of blubber is considered metabolically the most active in incorporating FAs from recently consumed food (Koopman et al. 1996; Budge et al. 2004). Confirming the same functional vertical layers and transition zones of blubber in the grey seals of this study as were found earlier in the ringed seal suggests that the vertical blubber layering is likely also found in other pinnipeds of the same size. Realizing this gave us the justification to next compare the FA characteristics and compositional variability in these three layers: outer blubber, middle blubber and inner blubber.

Saturated and monounsaturated fatty acids stratify extensively: polyunsaturated fatty acids less

That the outer blubber serves as the thermoregulatory adipose tissue and the middle and inner layers as energy-storing depot is manifested in the heterogeneity of biochemical properties found at different depths of the blubber (Liwanag et al. 2012). In accordance with previous pinniped studies (e.g. Bradshaw et al. 2003; Strandberg et al. 2008; Guerrero and Rogers 2017), we found that the outer blubber of the grey seals was enriched with MUFAs (especially 16:1n-7 and 18:1n-9) and contained lower relative concentrations of SFAs than the middle and inner layers. Compared to the pronounced vertical stratification patterns of the MUFAs and SFAs, the degree of stratification of PUFAs was moderate. Despite that PUFAs of tissue lipids are regarded as fluidity increasing components, only the shortest PUFAs, 18:2n-6 and 18:3n-3, had slightly (but statistically significantly) higher mol% values in the poikilothermal outer blubber than in the middle or inner blubber. The average levels of the longer chain and more highly unsaturated FAs remained unchanged through the depth of the blubber (except 20:5n-3, which was less abundant in the outer blubber than in the other layers). Since the lipids of the prey in the Baltic Sea are not especially rich in MUFAs but the PUFAs and SFAs dominate (Keinänen et al. 2017; Tverin et al. 2019), the high MUFA contents of the outer blubber of the seal can be seen as an adaptive response to the varying and cooler tissue temperature of outer blubber. The MUFA enrichment in

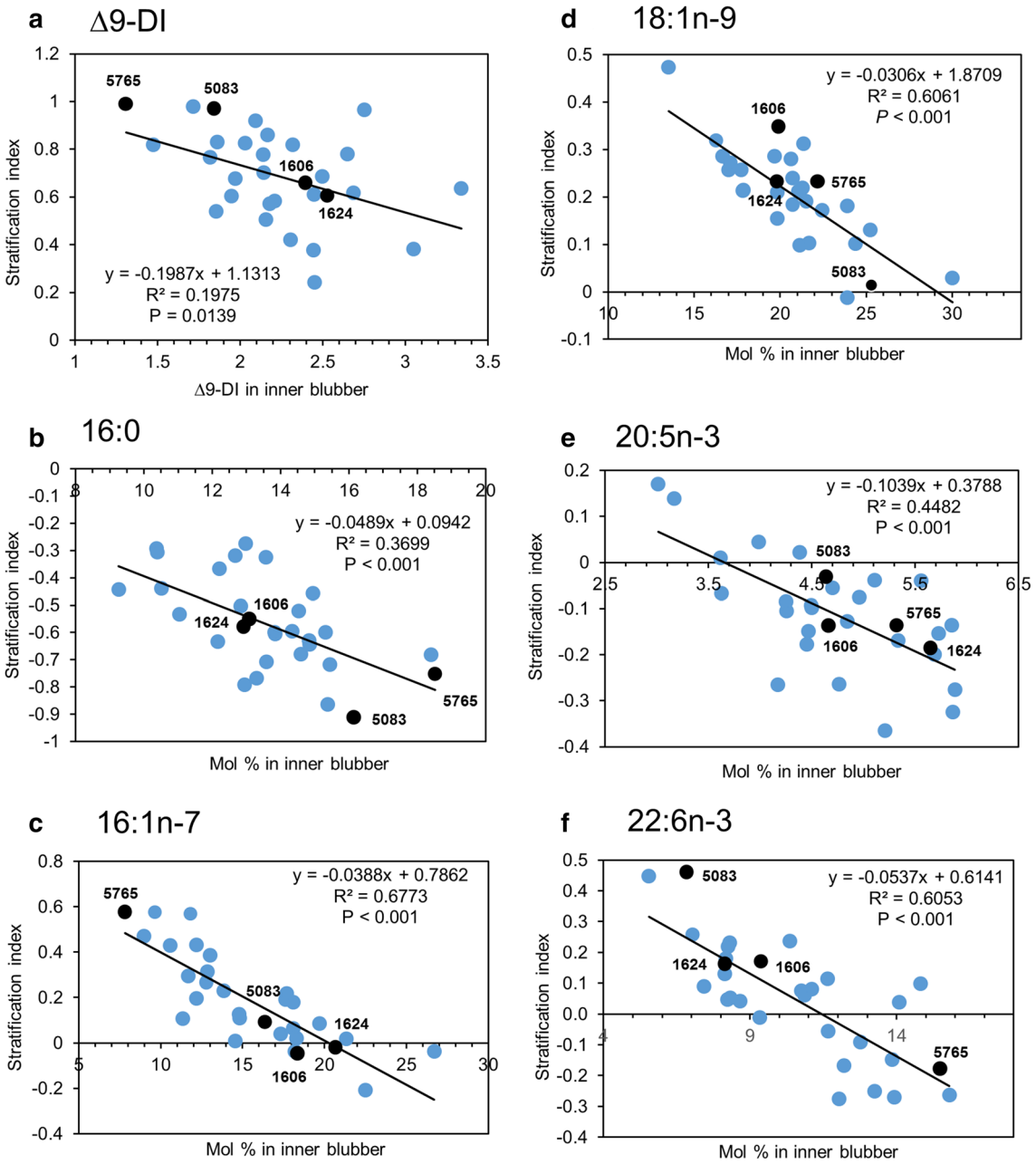


Fig. 9 Stratification index (SI, outer versus middle blubber) for **a** Δ9-DI or individual fatty acid **b** 16:0, **c** 16:1n-7, **d** 18:1n-9, **e** 20:5n-3, **f** 22:6n-3 mol% of the studied adult male grey seal ($n=30$) blubber plotted against the corresponding inner blubber value. This procedure yielded lines (with shown R^2 and P values) which either crossed the x axis or not. The x axis crossing points were determined by solving the line equation for when $y=0$, and these values stood for the FA proportions in grey seal inner blubber, which does not drive difference in its relative concentrations between the outer and middle blubber layer. Complete vertical fatty acid profiles (for the five main components) for the four individuals marked by black symbol with an identification code and having varying degree of SI are shown in Fig. ESM 2

the outer blubber could be explained by the ubiquitous and constant de novo synthesis or supply of the precursors 16:0 and 18:0, and their subsequent efficient Δ9-desaturation. The activity of cell membrane-bound Δ9-desaturase (SCD), an enzyme known to be responsible for the insertion of the first double bond to SFAs, is increased at low tissue temperatures (Nakamura and Nara 2004). Therefore, the high ratio of MUFAs to SFAs in superficial tissues of different mammalian species (Käkelä and Hyvärinen 1996) as well as in poikilothermic fish (Käkelä et al. 2008) could be consequences of elevated Δ9-desaturase activity. Although the enzyme activity has never been directly determined in

Table 3 Generalized linear model (GLM) analysis of the effect of fatty acid mol% in inner blubber and the seal age on the stratification index (SI, for outer versus middle blubber) of that specific fatty acid across the blubber layers in adult male grey seals ($n = 30$)

Fatty acid	Effect	*B*	Std. Error	LWC	UWC	Wald χ^2	*df*	Sig.
14:0	Intercept	− 0.033	0.141	− 0.309	0.244	0.053	1	0.817
	FA	− 0.082	0.034	− 0.149	− 0.014	5.653	1	0.017
	Age	NS	NS	NS	NS	NS	NS	NS
	FA × Age	NS	NS	NS	NS	NS	NS	NS
14:1n-5	Intercept	1.168	0.105	0.963	1.373	124.948	1	< 0.001
	FA	− 0.728	0.200	− 1.120	− 0.336	13.273	1	< 0.001
	Age	NS	NS	NS	NS	NS	NS	NS
	FA × Age	NS	NS	NS	NS	NS	NS	NS
16:0	Intercept	− 0.011	0.149	− 0.302	0.281	0.005	1	0.942
	FA	− 0.050	0.010	− 0.070	− 0.029	22.538	1	< 0.001
	Age	0.009	0.003	0.002	0.015	7.403	1	0.007
	FA × Age	NS	NS	NS	NS	NS	NS	NS
16:1n-7	Intercept	1.240	0.170	0.905	1.575	52.760	1	< 0.001
	FA	− 0.067	0.011	− 0.089	− 0.046	37.707	1	< 0.001
	Age	− 0.039	0.013	− 0.065	− 0.013	8.458	1	0.004
	FA × Age	0.002	0.001	0.001	0.004	8.193	1	0.004
18:0	Intercept	− 0.174	0.132	− 0.433	0.084	1.746	1	0.186
	FA	− 0.333	0.090	− 0.510	− 0.156	13.623	1	< 0.001
	Age	NS	NS	NS	NS	NS	NS	NS
	FA × Age	NS	NS	NS	NS	NS	NS	NS
18:1n-9	Intercept	0.728	0.079	0.574	0.882	85.795	1	< 0.001
	FA	− 0.025	0.004	− 0.032	− 0.017	43.260	1	< 0.001
	Age	NS	NS	NS	NS	NS	NS	NS
	FA × Age	NS	NS	NS	NS	NS	NS	NS
18:1n-7	Intercept	0.504	0.132	0.246	0.762	14.618	1	< 0.001
	FA	− 0.105	0.028	− 0.159	− 0.051	14.388	1	< 0.001
	Age	NS	NS	NS	NS	NS	NS	NS
	FA × Age	NS	NS	NS	NS	NS	NS	NS
18:2n-6	Intercept	0.488	0.082	0.327	0.648	35.448	1	< 0.001
	FA	− 0.064	0.020	− 0.103	− 0.025	10.378	1	0.001
	Age	NS	NS	NS	NS	NS	NS	NS
	FA × Age	NS	NS	NS	NS	NS	NS	NS
18:3n-3	Intercept	0.434	0.135	0.170	0.698	10.371	1	0.001
	FA	− 0.117	0.059	− 0.232	− 0.002	4.004	1	0.045
	Age	NS	NS	NS	NS	NS	NS	NS
	FA × Age	NS	NS	NS	NS	NS	NS	NS
18:4n-3	Intercept	0.254	0.091	0.075	0.433	7.721	1	0.005
	FA	− 0.235	0.057	− 0.346	− 0.123	17.010	1	< 0.001
	Age	NS	NS	NS	NS	NS	NS	NS
	FA × Age	NS	NS	NS	NS	NS	NS	NS
20:1n-7	Intercept	− 0.331	0.134	− 0.593	− 0.069	6.151	1	0.013
	FA	− 0.347	0.139	− 0.619	− 0.075	6.244	1	0.012
	Age	NS	NS	NS	NS	NS	NS	NS
	FA × Age	NS	NS	NS	NS	NS	NS	NS
20:4n-6	Intercept	0.361	0.121	0.125	0.598	8.960	1	0.003
	FA	− 0.566	0.140	− 0.841	− 0.291	16.302	1	< 0.001
	Age	NS	NS	NS	NS	NS	NS	NS
	FA × Age	NS	NS	NS	NS	NS	NS	NS
20:5n-3	Intercept	0.379	0.101	0.181	0.577	14.057	1	< 0.001
	FA	− 0.104	0.021	− 0.145	− 0.063	24.365	1	< 0.001
	Age	NS	NS	NS	NS	NS	NS	NS

Table 3 (continued)

Fatty acid	Effect	*B*	Std. Error	LWC	UWC	Wald χ^2	*df*	Sig.
	FA × Age	NS	NS	NS	NS	NS	NS	NS
22:5n-6	Intercept	0.596	0.163	0.277	0.915	13.404	1	< 0.001
	FA	− 2.571	0.750	− 4.042	− 1.101	11.753	1	0.001
	Age	NS	NS	NS	NS	NS	NS	NS
	FA × Age	NS	NS	NS	NS	NS	NS	NS
22:6n-3	Intercept	0.614	0.086	0.445	0.783	50.628	1	< 0.001
	FA	− 0.054	0.008	− 0.069	− 0.038	46.012	1	< 0.001
	Age	NS	NS	NS	NS	NS	NS	NS
	FA × Age	NS	NS	NS	NS	NS	NS	NS

B coefficient of the dependency, *LWC* lower 95% Wald confidence interval, *UWC* upper 95% Wald confidence interval, *Wald* χ^2 Wald Chi squared test value

pinniped blubber, the indirect proxy of its function, the Δ9-DI (calculated by dividing its MUFA products by SFA precursors in the tissue) has been shown to be very high (7–14) if only the most superficial subsamples of pinniped blubber were addressed (Käkelä and Hyvärinen 1996; Strandberg et al. 2011) but much less (2–4) if samples spanning the outer half of full depth or the whole full depth of the blubber column was studied (Best et al. 2003; Wheatley et al. 2007; Thiemann et al. 2008; Liwanag et al. 2012). Likewise, in the current study, the Δ9-DI was high (on average 7.2 for the 0–3-mm sample and 5.1 for the 3–6-mm sample) in the outer blubber of the grey seal, while the value was low (on average 2.2) in the inner blubber (Fig. 8c). In addition, the SI of Δ9-DI of these males was explicitly positive (Fig. 9a), which suggests that Δ9-DI is one major driver of the blubber vertical FA stratification.

It is surprising that the outer blubber Δ9-DI of the grey seals of this study showed a slight negative correlation with the age of the seal, while in ringed seals, the outer blubber Δ9-DI was clearly positively correlated with the age of the individual (Fig. 8, Käkelä and Hyvärinen 1996; Strandberg et al. 2011). Since the ringed seal is an Arctic species adapted to more northern habitats than the grey seal and also the body size of the ringed seal is smaller than that of the grey seals of the same age, maintaining sufficient thermoregulation may require better developed structural and biochemical adaptations of superficial adipose tissue improving with age. The few data available suggest that the older male ringed seals have thicker blubber layer and steeper temperature gradients in the blubber with lower skin temperatures than younger and leaner ones (Irving and Hart 1957; Taugbol 1982; Liwanag et al. 2012). We are not aware of any studies on the age dependency of grey seal skin temperature. However, infrared thermographic recordings of captive juvenile harbor seal (*Phoca vitulina*) and adult female Steller sea lion (*Eumetopias jubatus*) surface temperature indicated that the harbor seals, being younger and smaller, had slightly higher and more variable surface temperatures than the adult sea lions (Mellish et al. 2013). Thus, in general, large adult seal specimens would be able to maintain lower surface temperatures (reducing heat loss), which would require higher Δ9-DI in their superficial blubber FAs. In the grey seals of this study, however, the surface water temperatures on the day of collection had no statistically significant effect on the seal outer blubber Δ9-DI. This is understandable since Baltic grey seals were reported to prefer foraging depths of 11–40 m surrounding haul-out sites (Sjöberg and Ball 2000) and e.g. in 2012, the SD 27 area buoy (Huvudskär) recorded from the depth of 40–45 m close to 4 °C temperatures during all the sampling dates. This means that seasonal variation in blubber Δ9-DI would not be beneficial and would limit foraging trips below thermocline. Apparently, the outer blubber Δ9-DI does not show short-term variation but its degree may rather be evolutionarily adapted to the average temperature of the habitat.

The questions remain why PUFA total proportions were not enriched in the poikilothermic outer blubber and showed no age dependency? This probably requires addressing (i) the outer blubber characteristics at the lipid molecule level and also (ii) the effects of the Δ9-desaturase and its product the FA 16:1n-7 on adipose tissue kinetics. First (i), Strandberg (2012) found that the ratio of the membrane phospholipids, sphingomyelin (SM) to phosphatidylcholine (PC), was high in the outer blubber and low in the inner layer. Likewise, the ratio between cholesterol and total phospholipids was high in the outer blubber and low in inner blubber layer. The high SM and cholesterol contents of outer blubber convey information on the mechanical rigidity of the adipocyte membranes. The acyl chains in SM are either MUFAs or SFAs (but not PUFAs), leading to tight membrane lipid packing. Further, membrane SM and cholesterol have high structural compatibility and strong short-distance attraction (Slotte 2016). Importantly, the lipids at the same time reject PC and other phospholipids with PUFAs (Wassall and Stillwell 2009). The richer SM and cholesterol contents give mechanical strength to the outer blubber but create a

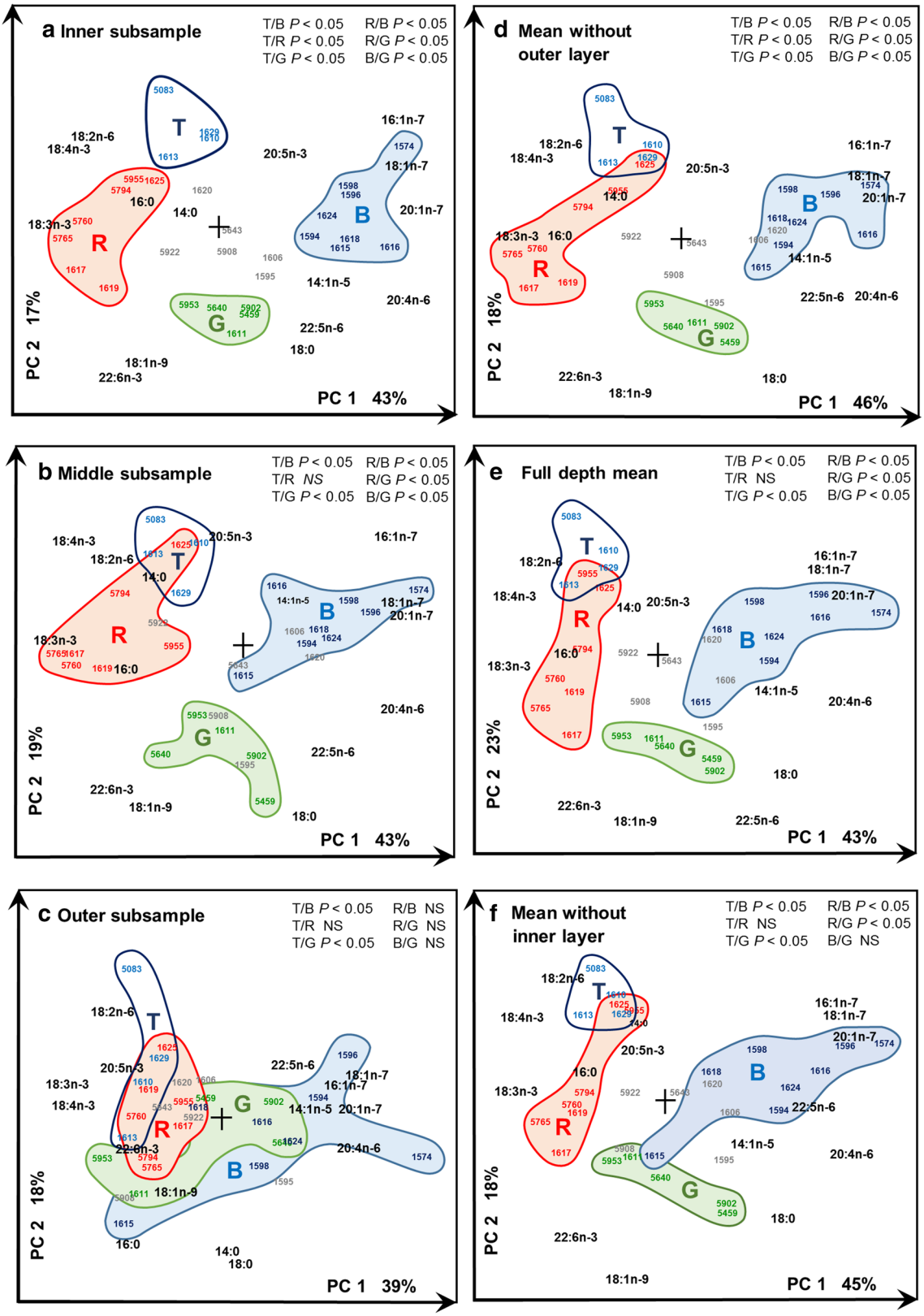
compatibility issue with PUFA-containing phospholipids. This may be one factor limiting the proportion of PUFAs in the outer blubber total FA pool serving as a source of precursors for the membranes. Second (ii), it has been found that high Δ9-desaturase activity promotes lipogenesis in mammalian tissues (Hodson and Fielding 2013). In pinniped outer blubber, this property may allow to maintain this from a thermoregulatory point of view the most important part of the blubber. Losing the insulating capacity of the outer blubber layer would inevitably lead to non-tolerable costs in body energy budget. The evident specialization of the outer blubber for the thermoregulatory role does not allow for non-selective dietary FA incorporation and thus the middle and inner blubber layers are likely to be the sites that reflect diet better. Indeed, the middle and inner blubber layers showed statistically significantly larger deviations of FA mol% values compared to the outer blubber. Still, in general, the magnitude of the FA mol% deviations differed the most between the outer and inner blubber, suggesting that the inner layer is the immediate location for dietary FA incorporation. We next tested the logic hypothesis that strong vertical stratification is found in blubber column only if the dietary FA mixture is very different from the endogenously regulated optimal composition of the outer blubber favouring MUFAs. The prerequisites for testing the idea were favourable since we (i) possessed a prey fish reference library including the FA profiles of 11 key prey fish species in the Baltic and (ii) had found that the individual feeding areas and prey types of the adult male grey seals were observable in their inner and middle blubber FA compositions (Tverin et al. 2019).

Mismatch between dietary fatty acids and the fatty acid preferences of outer blubber drives the vertical fatty acid stratification

Conducting GLM regression analysis indicated that the degree of stratification (SI) was largely dependent on the inner blubber FA composition, whereas only the SI of 16:0 and 16:1n-7 were influenced by age (Table 3). The curves revealed, for example, which MUFA mol% values in the inner blubber are high enough not to induce the enrichment of the specific MUFAs toward the outer layer. Thus, 16:1n-7 does not enrich in the outer blubber of the adult male grey seal if its mol% in inner blubber (reflecting dietary supply) is over 20 mol%, and 18:1n-9 does not enrich in outer blubber if the inner blubber level is over 30 mol% (Fig. 9c, d). For the main PUFA 22:6n-3, this dietary level not requiring stratification was about 12 mol% (Fig. 9f; vertical stratification of 22:6n-3 and its dependency on the inner blubber proportion of the FA was revealed only in intra-individual comparisons of subsamples but was masked by individual variability when comparing the average levels of the seals). It is likely that also in other pinniped species the degree of vertical FA stratifications is affected in similar fashion by the supply of dietary FAs incorporated via inner blubber to middle blubber. Strandberg et al. (2008) showed large individual variations in the vertical FA profiles of blubber in a study on Arctic ringed seal, the vertical FA curves of which infer similar principles ruling in ringed seal blubber as in the grey seal. In our opinion, the high MUFA contents in the inner blubber due to the high dietary supply mentioned above already meet the tissue-specific evolutionarily set demands of the thermoregulatory outer blubber layer and, therefore, no stratification is needed. In contrast, clear compositional changes are found at the depth of 0–15 mm from skin if PUFAs together with SFAs dominate the dietary FA supply. In conclusion, mismatch between dietary FAs and outer blubber FA demand set by environmental adaptation drives the vertical FA stratification.

Implications of the blubber vertical stratification of fatty acids on dietary studies

The increasing use of chemical tissue markers as a proxy of the diet of aquatic top predators (Budge et al. 2006; Iverson et al. 2007; Haug et al. 2017) and the increasing awareness of the uneven vertical stratification of these markers in the adipose tissue of the animals necessitates special attention to be given to the technique of tissue sampling for dietary studies (Strandberg et al. 2008, 2011; Guerrero et al. 2016, Bourque et al. 2018). Assuming that the composition of the outer blubber layer is endogenously controlled in pinnipeds, and that the inner and middle blubber layers are metabolically more active (Thiemann et al. 2004; Strandberg et al. 2008, 2011; Liwanag et al. 2012; Guerrero et al. 2016), it is valid to question whether the outer blubber layer should be included in the FA analyses of the tissue. In the current work, we analysed the representative subsamples of the three blubber layers for FAs separately, as well as the average FA composition of the whole blubber column. In addition, the effect of leaving the outermost 15 mm or alternatively the innermost 9 mm layer of the blubber out from the averaging was studied (Fig. 10). We found that the single representative inner blubber subsample (Fig. 10a) had a clearly better power to separate the investigated individuals according to the presumed diets than the outer blubber subsample (Fig. 10c), which had the worst separation power among all the tested blubber samples when the same set of variables including SFAs and MUFAs was used. Despite that pinniped blubber has been reported to be compositionally heterogeneous and that the importance of considering the vertical FA stratification has been underlined (Thiemann et al. 2004; Strandberg et al. 2008, 2011), any recommendations to omit a certain layer when studying pinniped blubber to assess diet have not yet been proposed. Since numerous dietary studies of pinnipeds have been conducted using the whole


a Inner subsample
T/B P < 0.05 R/B P < 0.05
T/R P < 0.05 R/G P < 0.05
T/G P < 0.05 B/G P < 0.05
PC 2 17%
PC 1 43%
d Mean without outer layer
T/B P < 0.05 R/B P < 0.05
T/R P < 0.05 R/G P < 0.05
T/G P < 0.05 B/G P < 0.05
PC 2 18%
PC 1 46%
b Middle subsample
T/B P < 0.05 R/B P < 0.05
T/R NS R/G P < 0.05
T/G P < 0.05 B/G P < 0.05
PC 2 19%
PC 1 43%
e Full depth mean
T/B P < 0.05 R/B P < 0.05
T/R NS R/G P < 0.05
T/G P < 0.05 B/G P < 0.05
PC 2 23%
PC 1 43%
c Outer subsample
T/B P < 0.05 R/B NS
T/R NS R/G NS
T/G P < 0.05 B/G NS
PC 2 18%
PC 1 39%
f Mean without inner layer
T/B P < 0.05 R/B P < 0.05
T/R NS R/G P < 0.05
T/G P < 0.05 B/G NS
PC 2 18%
PC 1 45%

◂**Fig. 10** PCA biplots of the blubber fatty acid composition of adult male grey seals ($n=30$) using different sampling procedures. The PCA biplots of **a** inner, **b** middle and **c** outer blubber subsamples are presented, as well as PCA biplots using the **d** mean values combining full inner and middle blubber layers (outer layer omitted), **e** mean values of the full-depth blubber, and **f** mean values combining full middle and outer blubber (inner layer omitted). The 15 main fatty acids were used as loadings. Paired SIMCA tests of the statistical significance of the compositional differences ($P<0.05$) are reported as inserts in the plots. Groups (Table 1) formed according to PCA dendrograms are: *T* trawl, *B* blue, *R* red, *G* green, and the individuals coded grey represent the "Remaining" group. *NS* non-significant. Numbers indicate individual seal identification codes

blubber column, we also investigated the separation power of the mean FA profile of the whole blubber column using PCA and subsequent clustering. When comparing the inner blubber subsample (Fig. 10a) and the average full depth composition (Fig. 10e), the separation power of the average composition was only slightly weaker; five out of six paired comparisons of the test groups performed by SIMCA remained statistically significant, which indicates that using the sample representing the whole blubber column still holds the criterions for this kind of studies, although the inclusion of the outer blubber layer FAs has a hampering effect on separation power (Fig. 10f). This adverse impact of the outer blubber FAs in a dietary study became clear in the comparisons with the blubber column samples, which averaged the FA profiles of all the inner and middle subsamples (but omitted the outermost 18 mm of blubber), or averaged the middle and outer layer FAs (but omitted the innermost 9 mm of blubber). If the FAs of the outer blubber layer were included, the power of separating individuals according to the test groups decreased. However, if the outer blubber was omitted, the sample averaging the inner and middle blubber FAs provided equally good test group separations as the representative single subsample of the inner blubber. This speaks for omitting the outer endogenously controlled layer from the samples used for dietary studies or alternatively finding ways to diminish the influence of the endogenous metabolism on the FA profile (which we tried by omitting the SFA and MUFA components) and exclusively using as loadings for PCA the diet-derived PUFAs. This approach improved the separation power and can be regarded as an option when sampling of the whole blubber column with the innermost layers is not possible, e.g. when studying marine mammals with very thick blubber, allowing to take biopsy samples from the outer blubber only.

Comparison of the current grey seal data with our earlier data on ringed seals (Käkelä and Hyvärinen 1996; Strandberg et al. 2008) showed a remarkable difference in the age dependency of the outer blubber MUFA enrichment between the species (Fig. 8). Thus, including the outer blubber into the samples might bring in compositional variation not originating from the diet. A further reason that supports the omission of the outer blubber layer in diet assessment studies is that the thickness of the middle blubber layer varies according to the energy status of the seal, which is seen in the current grey seal data and was also apparent in our earlier ringed seal data (Strandberg et al. 2008). Thus, the contribution of the outer blubber FAs to the full depth average FA composition is pronounced in the lean individuals but much less in the individuals having thick blubber. Such resulting deviation in average blubber FA composition, which is not due to the diet, can be avoided by removing the outer blubber from the samples used for a dietary study. In case only outer blubber layer can be sampled, then the FA profiling could be improved by selecting for analysis only PUFAs that cannot be de novo-synthesized. The large compositional divergence of the outer and inner blubber also suggests that the blubber FA composition of severely fasted individuals, in which the outer blubber dominates the full-depth average sample, is not optimal to assess diet. In addition, during a long-term fast, the FAs in the remaining thin thermoneutral layer of blubber (beneath the thermoregulatory outer blubber) are selectively mobilized and the remaining composition poorly reflects the diet (Raclot 2003). Thus, the data of this study apply to individuals with positive nutritional status and having sufficiently thick blubber, which allows vertical stratification.

Practical implications of sampling technique

Provided that the individuals used for diet assessment have not been fasting, (i) a representative subsample of the inner blubber or (ii) a full-depth sample from which the outer blubber layer has been removed appear to indicate the seal diet the best. Although both of these sample types served well in our grey seal study of adult males, it should be recalled that if the animals have variable diets with large temporal shifts, the inner blubber subsample and the average sample covering the inner and middle blubber are proxies of diets with different time windows. If the very recent diet is the target, then the inner blubber subsample should be focused on, and if the average diet of months is of interest, the sample should include the full inner and middle layers of blubber (Tverin et al. 2019). Apparently, outer blubber samples should be used with care and with selected FA variables. In pinnipeds, the outer blubber may decrease the sensitivity of the method, which should be taken into account when deciding on (i) feasible sampling procedures or (ii) the principles for data analysis, both possible to design to diminish the influence of endogenously produced or modified FAs. However, the outer blubber layer is of special interest when studying environmental adaptation of marine mammals, e.g. their temperature adaptation, and the remarkable vertical differences in blubber biochemistry and physiology (including

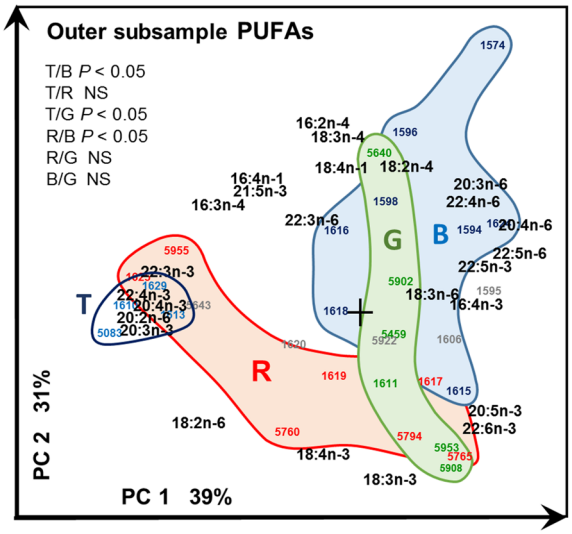


Fig. 11 PCA biplots for the polyunsaturated fatty acid (PUFA) composition in the outer blubber of adult male grey seals ($n=30$). All detectable PUFAs were used as loadings when comparing the outer blubber subsamples. Paired SIMCA tests of the statistical significance of the compositional differences ($P<0.05$) are reported as inserts in the plots. Groups (Table 1) formed according to PCA dendrograms are: *T* trawl, *B* blue, *R* red, *G* green, and the individuals coded grey represent the "Remaining" group. *NS* non-significant. Numbers indicate individual seal identification codes

enrichment of 16:1n-7 in the outer blubber) can possibly be utilized as a model when studying adipose tissue metabolism and dynamics from biomedical perspectives (Hodson and Fielding 2013; Houser et al. 2013; Wang et al. 2015; de Souza et al. 2018).

Omitting the outer blubber layer from the sample analysed for a dietary study improves the power of the FA marker technique when applied to the adipose tissue of small-size pinnipeds adapted to cold water. We also emphasize that the varying degree of vertical FA stratification in different individuals causes no uncertainty to dietary estimates when made using the inner and middle blubber FA profiles, no matter how different their FA composition is from the endogenously modified relatively uniform FA composition of the outer blubber. Despite the outer blubber FA profile did not indicate dietary differences in our model species, the grey seal equally well as the FA profiles of the deeper layers, its separation power in multivariate analysis could be improved using as variables only a selection of diet-derived FAs, a procedure potentially useful when inner blubber cannot be reached when sampling. In this work, we have established a clear hypothesis on the causes of the vertical FA stratification of pinniped blubber and proposed procedures for sampling of blubber to improve the accuracy of monitoring pinniped diet by FA analysis, which now waits for validation in different seal populations.

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Data availability statement The datasets during and/or analysed during the current study are available from the corresponding author on reasonable request.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval Samples were collected from ongoing national monitoring programmes of fish and seals in the Baltic Sea, according to relevant national and international guidelines, and did not require any separate permits from animal research ethics committees. Thus, all applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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