MALIN TVERIN

FACTORS AFFECTING THE FATTY ACID PROFILE OF ADIPOSE TISSUES USED TO ASSESS INDIVIDUAL DIETARY HISTORY OF GREY SEALS AND GREAT CORMORANTS IN THE BALTIC SEA

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Factors affecting the fatty acid profile of adipose tissues used to assess individual dietary history of grey seals and great cormorants in the Baltic Sea

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This too shall pass.
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List of original publications

This thesis is based on the following articles and manuscript, later referred to as Part I-III by their Roman numerals as listed here.


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Author contribution (I-III)

Malin Tverin contributed to planning, conducting experiments and data analysis. She wrote the first versions of the manuscripts, and edited them further with the coauthors, and is the corresponding author in the articles I and III.
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<tr>
<td>AA</td>
<td>Arachidonic acid, 20:4n-6</td>
</tr>
<tr>
<td>ACC</td>
<td>Acetyl-CoA-carboxylase</td>
</tr>
<tr>
<td>ALA</td>
<td>Alpha-linolenic acid, 18:3n-3</td>
</tr>
<tr>
<td>CoA</td>
<td>Coenzyme-A</td>
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<tr>
<td>DHA</td>
<td>Docosahexaenoic acid, 22:6n-3</td>
</tr>
<tr>
<td>DI</td>
<td>Desaturation index</td>
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<tr>
<td>EFA</td>
<td>Essential fatty acid</td>
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<tr>
<td>FA</td>
<td>Fatty acid</td>
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<tr>
<td>FADS</td>
<td>Fatty acid desaturase</td>
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<tr>
<td>FADS1</td>
<td>Fatty acid desaturase1 or Δ5 desaturase</td>
</tr>
<tr>
<td>FADS2</td>
<td>Fatty acid desaturase2 or Δ6 desaturase</td>
</tr>
<tr>
<td>FAME</td>
<td>Fatty acid methyl ester</td>
</tr>
<tr>
<td>FAS</td>
<td>Fatty acid synthase</td>
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<tr>
<td>FID</td>
<td>Flame ionization detector</td>
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<tr>
<td>GC</td>
<td>Gas chromatograph</td>
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<tr>
<td>GLM</td>
<td>General linear model</td>
</tr>
<tr>
<td>GPL</td>
<td>Glycerophospholipid</td>
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<tr>
<td>ICES</td>
<td>International Council for the Exploration of the Sea</td>
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<tr>
<td>LA</td>
<td>Linoleic acid, 18:2n-6</td>
</tr>
<tr>
<td>LCMUFA</td>
<td>Long-chain monounsaturated fatty acid</td>
</tr>
<tr>
<td>LDL</td>
<td>Low density lipoprotein</td>
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<tr>
<td>LPL</td>
<td>Lipoprotein lipase</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>MS</td>
<td>Mass spectrometer</td>
</tr>
<tr>
<td>MUFA</td>
<td>Monounsaturated fatty acid</td>
</tr>
<tr>
<td>nMDS</td>
<td>Non-metric multidimensional scaling</td>
</tr>
<tr>
<td>NMID</td>
<td>Non-methylene interrupted diene</td>
</tr>
<tr>
<td>PCA</td>
<td>Principal component analysis</td>
</tr>
<tr>
<td>Pcc</td>
<td><em>Phalacrocorax carbo carbo</em></td>
</tr>
<tr>
<td>Pcs</td>
<td><em>Phalacrocorax carbo sinensis</em></td>
</tr>
<tr>
<td>PUFA</td>
<td>Polyunsaturated fatty acid</td>
</tr>
<tr>
<td>RSD</td>
<td>Relative standard deviation, also known as Coefficient of variation, CV</td>
</tr>
<tr>
<td>SCD</td>
<td>Stearoyl CoA-desaturase or Δ9 desaturase</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation / Subdivision (of Baltic Sea ICES-areas)</td>
</tr>
<tr>
<td>SFA</td>
<td>Saturated fatty acid</td>
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<tr>
<td>SI</td>
<td>Stable isotope / Stratification index</td>
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<td>SIMCA</td>
<td>Soft independent modeling of class analogy</td>
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<tr>
<td>SM</td>
<td>Sphingomyelin</td>
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<tr>
<td>TAG</td>
<td>Triacylglycerol</td>
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<tr>
<td>VLDL</td>
<td>Very low density lipoprotein</td>
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Abstract

Growing populations of the piscivorous top predators the grey seal (Halichoerus grypus) and the great cormorant (Phalacrocorax ssp) in the Baltic Sea have created predator-fishery conflicts. Responsible mitigation measures require that correct and adequate information on the predator foraging habits is available. Adipose tissue fatty acid (FA) profiling is an established method for such dietary and food web studies since the FAs are transferred from the prey to predator tissues in a predictable way. However, sampling procedures and the way of using the FA profiles need to be optimized for each predator phyla to create globally applicable and reliable procedures for the diet assessment. One prerequisite is to build a reference library of the FA compositions of the available prey selection (fishes in the Baltic Sea), and the predator tissue metabolism and other factors affecting the tissue FA profiles need to be studied in order to avoid misinterpretation of results. We analysed tissue FA profiles of fishes, grey seals and great cormorants from the Baltic Sea area by gas chromatography, and used multivariate Principal Component Analysis (PCA) along with Soft Independent Modelling of Class Analogy (SIMCA) to analyse the multivariate data.

Pinniped blubber FA profile differs vertically through the blubber column from skin to muscle, which raises a question whether sampling depth causes discrepancy in the results. We found that the vertical FA stratification patterns show constant trends, but also large individual differences. All studied adult grey seal males (n = 30) had enrichment of monounsaturated FAs (MUFAs) in their outer blubber, whereas polyunsaturated FAs (PUFAs) did not show similar strong stratification pattern along the blubber column as the MUFAs did. The degree of vertical FA stratification did not depend on blubber thickness and was slightly age-dependent only for two FAs, 16:0 and 16:1n-7. In the absence of an effect caused by other factors such as sex, blubber thickness, capture area and water temperature, we found that the driver of the vertical stratification of MUFAs was the mismatch between the individually varying FA composition of the inner blubber, regarded as the main incorporation site of dietary FAs, and the outer blubber FA composition, endogenously enriched in MUFAs. This highlights the importance of choosing sampling protocols in which the outer blubber layer is omitted from the analysis, and preferably only the innermost blubber, which carries the most recent dietary information, is included to gain valid information of the diets of the seals. To evaluate the pros and cons of the FA profiling in comparison to other available methods, we analysed the blubber of 108 grey seal individuals collected from the Finnish and Swedish coast of the Baltic sea, and compared dietary information obtained by blubber FA analysis to the information from gut content hard part or DNA analyses, and tissue stable isotope (SI) ratios. We found that in a large data set, PCA of blubber FA profiles did not manage to clearly detect any dietary groups, which was likely due to the large individual variation in the foraging habits of the seal individuals. In smaller scale, however, blubber FA profiles distinguished groups of individuals with accurately known collection site and which likely had used the same type of prey for a longer period of time. This information is valuable since these individuals likely
cause the largest economical losses in the form of loss of catch and destroyed fishing gear, which in turn is the culprit to the risen seal-fishery conflict in the Baltic Sea.

The population of the avian top predator of the Baltic Sea, *i.e.* the great cormorant (including two subspecies *Phalacrocorax carbo sinensis* and *Phalacrocorax carbo carbo*), has also grown and caused local conflicts with fisheries. In the absence of studies of chemical tissue markers of the great cormorants of the Baltic Sea area, we analysed the subcutaneous knee adipose tissue of 77 cormorant individuals and found that the adipose tissue FA composition of the studied birds differed both spatially and temporally. Thus, the FA profiling is even more sensitive to indicate dietary differences in sea birds than in pinnipeds, presumably due to the high metabolic rate and short turnover time of the adipose tissue lipids and FAs. In addition to the spatial differences observed, which were likely due to locally differing diets, we also observed high relative amounts of ocean FA marker 22:1n-11 in the adipose tissue of the cormorants collected in Sundsvall in June 2017, which likely had got their 22:1n-11 from hatchery-reared salmonids fed ocean fish flour and oils. Since levels of certain FAs or their ratios are characteristic of specific dietary sources and since dietary FAs are assimilated in predator tissues in a predictable way, FA profiling is a useful tool for dietary studies both on individual and population level. To describe the variability of prey sources at population level, we calculated the relative standard deviations (RSD, *i.e.* SD/Mean) for the FA variables (the mean value of which exceeded 0.2 mol%, and thus excluded methodological variation) in the adult cormorant and adult seal individuals. The FA variables with the highest RSD values were the most useful markers of individual dietary differences in the population. We propose that the RSDs for FA variables could be used to create an “opportunistic feeding index”, providing information on the variability of prey of the studied population. The works that this thesis is based on show, that standardized sampling protocols that take into account tissue metabolism significantly, increase the power of FA profiling in indicating individual diets of aquatic top predators, and allow creating indices to describe population level dietary opportunism. The improved dietary information can be applied for monitoring the diets of aquatic top predators and for ecosystem-based management.
1. Introduction

1.1 Aquatic food webs

Characteristic to any food web is its levelled structure with producers such as protists, bacteria and algae found at lower trophic levels and consumers such as zooplankton at higher trophic levels, and, in many food webs, finally mammalian or avian top predators on the highest trophic level. All trophic levels in the food web are dependent on the dynamics of the ecosystem (Lindegren et al. 2011), which can have characteristics of the top-down and/or bottom-up regulation of populations (Lynam et al. 2017). The modes of regulation are influenced by environmental factors such as the nutrient input, temperature and salinity, anthropogenic factors and species community structure at different trophic levels, and alterations in these cause subsequent change in the fluxes of matter and energy through the trophic levels of the food web (Wulff et al. 1990, Reiss et al. 2014).

Essential for the survival of any organism is sufficient energy input. Prey availability as well as the nutritionally ideal chemical composition of prey determine the condition and fitness of an individual. Dietary lipids providing fatty acids (FAs) for fuel are a central carrier of chemical energy in aquatic food webs (Budge et al. 2006). For example, the n-3 polyunsaturated FAs (n-3 PUFAs) have been shown to affect fish development and growth (Strandberg et al. 2018). Changes in the productivity of the food web at lower levels lead to changes in prey availability or prey quality for higher trophic levels, finally manifested in different dietary supply of nutrients to top predators (Fig. 1) (Begon et al. 2005, Lindegren et al. 2011).

![Figure 1. A general scheme of the trophic levels in an aquatic food web. Energy and matter fluxes between trophic levels are dynamic and the primary producers on the lowest trophic levels set the ground for all higher trophic levels. Modified from Begon et al. 2005.](image)
Since the ecosystem dynamics are vulnerable to interference by various anthropogenic influences including climate change, the knowledge on intertrophic fluxes and relationships as well as identification of key prey and key consumers in a food web is a prerequisite for successful and accurate ecosystem-based management of populations and ecosystem risk assessment (Wulff et al. 1990, Casini et al. 2011). A detailed understanding of the interconnections in a food web is necessary for making predictions on the possible consequences of any occurring change at any trophic level of the ecosystem prone to natural and anthropogenic influence. Especially the identification of a predator’s key prey species is essential since, if possibilities of prey switching are limited, the fluctuations of key prey populations may cause fluctuations in predator population size (Reiss et al. 2014). Such changes may create conflicts with fisheries if the top predators increase their utilization of fish from fishing gear, which also is likely to increase the mortality of the predators that become bycaught in the fishing gears (Königson et al. 2013, Hansson et al. 2018). The species composition at different trophic levels of the food web determines the composition of biomolecules, especially that of the FAs, transferred in the food web. The changes in the food web biochemistry can be unexpectedly fast. For example, Lind et al. (2018) showed that the FA composition of Baltic herring (Clupea harengus membras), an important prey of the Baltic seals and seabirds, showed clear spatial north–south segregation 50 years ago, but currently the herrings of the north and south have relatively similar tissue FA compositions.

1.2 Fatty acids

1.2.1 Fatty acid structure

Fatty acids are neutral molecules consisting of a hydrocarbon chain ending in a carboxyl group. Three different nomenclatures; Δ- and n- or ω-nomenclature have been used to abbreviate the long full names. They all announce the length of the acyl chain, the number of double bonds and the locations of the double bonds in the acyl chain. When Δ-nomenclature announces the positions of double bonds counting from the carboxyl end, the n- or ω-nomenclatures in practical sense announce the place of the first double bond counted from the methyl end. Strictly, n-x tells the number of carbons in the “normal” alkyl chain in the tail of the chain. The nomenclature also assumes that the adjacent double bonds are separated by one methylene group (-CH₂-) (Tocher 2003). In this thesis, the n-nomenclature is used and FAs are announce as [carbon number]:[number of double bonds] n-[position of the first double bond calculated from the methyl end] (e.g. 22:6n-3) (Fig. 2).
Figure 2. Structure examples for a saturated fatty acid (18:0), monounsaturated fatty acid (18:1n-9) and polyunsaturated fatty acid (18:3n-3). The principles of n- (ω-) and Δ-abbreviations are demonstrated. Modified from Budge et al. 2006.

The structures of FAs determine their chemical and physiological properties, and, also, as demonstrated in this thesis work, tell about the origin of the FAs. Saturated FAs (SFAs) have no double bonds between the carbons of the acyl chain, whereas monounsaturated FAs (MUFAs) have one double bond, and PUFAs have two or more double bonds in the acyl chain (Fig. 2) (Budge et al. 2006). Commonly, FA chains with 14-24 carbons (later: C, e.g. C14-24) and their derivatives are found in aquatic animals, and the total number of existing FAs in nature likely reaches a high value, since up to 70 different FAs may be found in the tissues of any given organism (Iverson 2008). In tissues of mammals and birds, FAs are largely found attached to structural lipids e.g. in the glycerophospholipids (GPLs) of cell membranes, and to triacylglycerols (TAGs) of storage lipid droplets, that for example fill adipocytes (Ramírez et al. 2001). The metabolic modifications of FAs in vertebrate tissues are described in the next chapter.

1.2.2 Fatty acid synthesis and modification

The synthesis routes and modification mechanisms of FAs are very similar in different vertebrate species but their use vary according to the energy balance of the organism and large tissue specific differences in the activities of different responsible enzymes exists (Raclot 2003). FAs can be found “free”, e.g. in the bloodstream when they are transported bound to albumin. The free FAs appear as a result of lipase hydrolysis freeing them from adipose tissue during negative energy state or postprandially prior to incorporation into adipose tissue for storage. Large part of circulating FAs is found packed in the lipids of the lipoprotein particles in blood plasma. In mammals, de novo synthesis in liver yields mainly palmitic acid 16:0 (but minor amounts of other C14-18 straight and branched chain SFAs are also synthesized), and in vertebrates further enzymatic modifications may yield FAs up to C24 with different double bond contents (Gouillou et al. 2010). As trace components, even longer FAs can be commonly detected in animal tissues (Käkelä et al. 1995).
The liver cell cytoplasm is a major site for de novo synthesis of FAs in birds and mammals (Lehner & Kuksis 1996, Klasing 1998, Williams & Buck 2010). In short, FA synthesis includes multiple reactions in an enzymatic process starting from the conversion of acetyl CoA (first transported from mitochondria to the cytosol) into malonyl CoA in the cytosol by the acetyl-CoA-carboxylase (ACC). Acyl chains are then formed when the enzyme complex FA synthase (FAS) uses the acetyl-CoA as the main primer and adds two carbon atoms in a cyclic manner as repeated condensation reactions with malonyl-CoA (Reddy & Hashimoto 2001, Rangan & Smith 2002). Finally, this process yields the 16:0 as the main de novo product (and minor amounts of other SFAs with chain length C14-18) (Guillou et al. 2010). The de novo synthesized FAs are then further modified into their corresponding MUFAs and elongated to long-chain MUFAs (LCMUFAs) by elongase enzymes (Gouillou et al. 2010) (Fig. 3).

Figure 3. Schematic picture of the modification of the de novo-synthesized fatty acids. Modified from Guillou et al. 2010.

In addition to the de novo synthesized FAs, some specific FAs of long chain length are essential to vertebrates but cannot be synthesized by themselves since they are restricted in their ability to de novo synthesize so called “essential FAs” (EFAs), which are long-chain (C20-22) PUFAs such as arachidonic acid (AA, 20:4n-6), eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3). Hence the EFAs need to be exogenously acquired from diet either as their C18 precursors, α-linolenic acid (ALA, 18:3n-3) and linoleic acid (LA, 18:2n-6) to be further
modified by the animals, or they need to be acquired as the ready-made highly unsaturated C20-22 FAs (Sprecher 2000, Guillou et al. 2010). The modification of the precursor PUFAs includes alternating reactions of enzymatic desaturation and acyl chain elongation and sometimes additional partial shortening, as required for the synthesis of 22:6n-3 (Sprecher 2000, Salem et al. 1996). These processes yield C20-24 PUFAs of the n-3, n-6 and n-9 structural families, the synthetic steps of which are competitive between the families and in which the end products inhibit each other (Simopoulos 2016) (Fig. 4).

**Figure 4.** Schematic picture of the modification steps of the C18 precursors of the n-3, n-6 and n-9 families to the highly unsaturated, and in the case of n-3 and n-6 fatty acids, essential products. Modified from Sprecher et al. 2000.

The desaturation, *i.e.* introduction of double bonds into the acyl chain, is done by desaturase enzymes named after the site where they place the new double bond counted from the carboxyl group end. Birds and mammals are restricted in their ability to desaturate acyl chains beyond C9 from the carboxyl group end since they only have the stearoyl CoA-desaturation (SCD or Δ9-desaturase) enzyme, fatty acid desaturase 1 (FADS1 or Δ5-desaturase) and FADS2 (Δ6-desaturase) activities (Iverson 2008, Lee et al. 2016). Plants and algae, however, are equipped with Δ12- and Δ15-desaturases and can thus produce FAs with a double bond already at the sixth or third C counted from the methyl end, yielding 18:2n-6 and 18:3n-3, which are the
precurors of the longer and highly unsaturated EFAs (Sprecher 2000). In bird and mammalian tissues, the EFAs 20:4n-6, 20:5n-3 and 22:6n-3 are produced from the C18 PUFAs by acyl chain modifications between the carboxyl group end and existing double bonds: chain elongation, further desaturation and also peroxisomal chain shortening (the Sprecher’s Shunt, Sprecher et al. 2000). The lack of the Δ4-desaturase causes this complicated train of events until the final end-product EFAs of the n-3 and n-6 families, i.e. 22:6n-3 and 22:5n-6 are reached. Thus, the presence of EFAs is dependent on both the C18 PUFA precursor availability and intake, as well as on the ability to modify the precursors into the C20-22 long and highly unsaturated EFAs. It is important to note that this ability varies according to phyla, and for instance, is almost completely lacking in marine predatory fish due to their rich supply of 20:4n-6, 20:5n-3 and 22:6n-3 from their prey fish (Sargent et al. 1999, Tocher 2003). Thus, the most biopotent C20-22 PUFAs can also be acquired from diet as ready-made.

The structural modifications are regulated by competitive inhibition between the n-3, n-6 and n-9 FA family members. When the precursors are available in equimolar amounts, the structural modifications of n-3 PUFAs are generally preferred over the modifications of the n-6 precursors (Sprecher 2000, Schmitz & Ecker 2008). The n-9 PUFAs are produced in significant amounts from oleic acid 18:1n-9 only in the case of EFA deficiency (Uauy et al. 1999, Fig. 4). The activity of Δ5- and Δ6-desaturase enzymes is largely regulated by their substrate availability, i.e. dietary levels of the C18 PUFA precursors (Nakamura & Nara 2004). The activity of the Δ9-desaturase enzymes is also regulated by tissue temperature (Nakamura & Nara 2004) but also by dietary intake of different FAs, e.g. by the PUFA supply from the diet, which inhibits endogenous MUFA production by the Δ9-desaturase enzyme (Ntambi 1995). The Δ9-desaturase enzyme is active in low temperatures, as in superficial adipose tissues of aquatic mammals (Käkelä & Hyvärinen 1996), and its high activity is observed as a high MUFA/SFA ratio, where the de novo synthesized precursor SFAs 16:0 and 18:0 get low while their Δ9-desaturation products 16:1n-7 and 18:1n-9, respectively, increase their proportions.
1.3 Marine food web monitoring

1.3.1 Fatty acids in marine food web monitoring

Especially when studying marine food webs with the help of tissue chemical markers, carbohydrates and proteins are of lesser significance compared to lipids, which are the most energy-dense biomolecules and contain structural parts, the FAs, which are largely conserved during the trophic transfer from prey to predator (Iverson 2008). FAs are thus informative when studying dietary fluxes and are suitable as ecosystem monitoring molecules since i) they are differently biosynthesized and modified (elongated and desaturated) by organisms in different animal phyla, ii) they are not degraded in digestion (Budge et al. 2006) and iii) they are stored in long-term energy reservoirs such as adipose tissue and thus accumulate over long periods of time (Iverson 2008). Carbohydrates and proteins do not possess these from the monitoring point of view beneficial traits.

While primary producers are able to synthesize complex FA structures, vertebrates are more restricted in their de novo capacity of synthesis, and produce mostly the saturated 14:0, 16:0 and 18:0 as well as their Δ9-desaturated products 14:1n-5, 16:1n-7 and 18:1n-9. Phytoplankton and seaweeds synthesize C14-24 FAs de novo, and among these organisms the algae can produce the very long C20-24 PUFAs. Copepod zooplanktons are able to synthesize LCMUFAs and fatty alcohols that they store as wax esters. These wax esters harbor fatty acids derived from de novo synthesis and theoretically, these wax esters can be oxidized to LCMUFAs (Sargent 1976). Benthic mollusks are equipped by rare desaturation enzymes and are thus capable of producing non-methyl interrupted (NMI) FAs, or non-methyl interrupted dienes (NMIDs), which have consecutive double bonds in their acyl chains, a phenomenon not commonly observed in other animal or plant phyla (Monroig et al. 2013). Thus, specific FAs characterize certain animal phyla or certain biotopes or geographical regions rich in these organisms. For example, the LCMUFA 22:1n-11 is a characteristic of ocean ecosystems (Dalsgaard et al. 2003, Wold et al. 2011) whereas 18:2n-6, 18:3n-3 and 20:4n-6 are characteristic of freshwater biotopes (Ågren et al. 1987, Keva et al. 2019). Odd- and branch-chain FAs, on the other hand, are produced by bacteria, e.g. in aquatic sediments (Perry et al. 1987).

In a certain population of animals of the same species, the individuals can be expected to possess similar capacity to synthesize and modify FAs derived from de novo synthesis and theoretically, the representatives of certain species on the same diet can be expected to have a similar FA composition in their tissues (Thiemann et al. 2007). Hence, the occurrence of certain FAs in the tissues of a predator not typical for the food web in that specific region indicates exceptional dietary factors bringing variability to the tissue FA composition on an individual level. The variation could arise from dietary FAs that originate from an unexpected trophic level provided by human aquaculture activities, or from an entirely different geographic region, accessible for free-ranging predators.

The adipose tissue FAs of marine free-ranging top predators are valid proxies in food web studies since long-term information on individual foraging habits and prey selection from geographically large areas as well as information on the entire...
food web, is stored in adipose tissue lipids of the predators (Cooper et al. 2005, Budge et al. 2006, Frayn et al. 2006). The FA profile changes in predator adipose tissue may reflect not only a change in prey availability, but also an underlying change in food web structure. However, to avoid misinterpretations, it is essential to know the physiology of the predator adipose tissue, especially the possible selective metabolic mechanisms affecting the incorporation of dietary FAs into the tissue. These would make the predator FA profiles not to exactly match those of the prey items (Grahl-Nielsen et al. 2011).

1.3.2 Gut content hard parts and DNA analysis, and tissue stable isotope analysis

Diet estimations of free-ranging animals can be made by using so called traditional methods, i.e. by examining undigested prey hard part remains, and DNA-based methods examining DNA remains, from the digestive tracts, faeces or regurgitates of the animals (Leopold et al. 2001, Barrett et al. 2007, Bowen & Iverson 2012). The drawback of these methods is the short time window since only the very recent diet is revealed and thus frequent, more long-term foraging habits cannot be observed. In traditional diet analysis, digestive erosion may cause bias if not accounted for (Jobling & Breiby 1986) and prey items with less, or even no, identifiable remains are easily underrepresented in the results, e.g. when only soft tissue have been ingested from large fish such as salmonids. Both traditional and DNA-based methods however, can enable determination of prey at species-level which is information that cannot be obtained by tissue FA analysis.

Tissue stable isotope (SI) chemical elements are also used in dietary studies and carry information on the trophic level and geographical origin of the prey (Dalerum & Angerbjörn 2005). The method is based on the general presence of elements e.g. $^{12}\text{C}$, $^{14}\text{N}$ and $^{32}\text{S}$, and their heavier isotopes $^{13}\text{C}$, $^{15}\text{N}$ and $^{34}\text{S}$ in the prey and predator, and on the predictable enrichment of the heavier isotopes in every step of trophic transfer, as the light isotopes are preferably excreted (Hobson 1999). Thus, the change in heavy versus light isotopes in the tissues of the animal function as a marker of trophic level (Sinisalo et al. 2008, Newsome et al. 2010). Moreover, the ratios of the SIs vary geographically and according to biotopes. The SI values in the predator tissues were normalized against Vienna PeeDee belemnite (VPDB) for carbon, atmospheric N2 (AIR) for nitrogen, and Vienna Canon Diablo Meteorite Troilite (V-CDT) for sulphur, and finally the trophic transfer is expressed as 1.3-5.3 promille (‰) per trophic level and denoted as e.g. $\delta^{13}\text{C}$ (Bond & Hobson 2012).

1.4 Seal and bird adipose tissue FAs as proxies for dietary FA intake

Blubber is a vascularized, collagen-containing adipose tissue type encountered subcutaneously in marine mammals and even in some marine birds like penguins. It has a dual role in providing thermoregulation and an on-board energy storage. Previous studies on small pinnipeds suggest (Strandberg et al. 2008; 2011) that blubber is metabolically and compositionally stratified into vertical layers, and that warm deeper layers are metabolically active while the cool superficial layers are less efficiently hydrolysed for energy metabolism, a phenomenon related to tissue temperature (Irving & Hart 1957). The use of blubber tissue as dietary proxy to make
estimations on marine mammal diet is based on its overall low FA turnover rates, and thus on the long time period from which dietary information can be retrieved (Budge et al. 2004, Strandberg et al. 2008; 2011). Blubber thickness varies individually according to the animal’s energy balance and the inner and middle layers are metabolized first as an animal enters negative energy balance (Hall et al. 2001, Bäcklin et al. 2011).

Bird knee adipose tissue is a safe, standardized sampling site that allows even for non-lethal catch and release sampling (Iverson et al. 2007, Käkelä et al. 2010, Owen et al. 2010, Rocha et al. 2016). This superficial adipose tissue has a FA composition which is alike the composition in the adipose tissue around the gastrointestinal tract and in the body cavity (Abrahamsson 2016). Similarly to the pinnipeds, seabird adipose tissue also carries information on dietary habits over a longer time period than possible to address by studying digestive tract contents, pellets or regurgitates. The time window of FA-based dietary information is a few weeks (Wang et al. 2010) due to the fast turnover of the avian adipose tissue, a characteristic due to the high metabolic rate of birds. Information from a longer time period would require the ability to store large amounts of adipose tissue, which is not the physiological property employed by avians due to the hampering effect excessive weight has on flying.

In mammals and therefore also pinnipeds, the uptake of dietary FAs from the gut lumen yields chylomicronal transport of dietary TAGs and cholesterol into the lymphatic circulation, where after they join the blood circulation. From the blood circulation, FAs may be hydrolysed from TAGs directly to tissues for energy or storage by the enzyme lipoprotein lipase (LPL) located in e.g. muscle or adipose tissue endothelial membranes. The chylomicron remnants (consisting of small amounts of cholesterol, TAGs and fat-soluble vitamins) will then be transported to the liver (receptor-mediated, receptor not shown in image) for further use, e.g. for energy production in β-oxidation, or for further FA modification. The mammalian lipid metabolism continues in the liver with the synthesis of very low density lipoprotein (VLDL) particles, which contain de novo-synthesized FAs, diet-derived and modified FAs and cholesterol. The VLDLs are secreted from the liver to the bloodstream, where they transport their FAs to peripheral tissues and adipose tissue. The delivery of the FAs is again mediated by tissue cell surface LPL, and after delivery of FAs to energy demanding tissues, VLDL particles are classified as low density lipoprotein (LDL) particles, which deliver their FA content to peripheral and adipose tissues via cell surface LDL-receptors, after which the LDL particle returns to the liver for further repeated metabolic cycles (Fig. 5a). In avians, however, the dietary fat-carrying lipoprotein particles, called portomicrons, are transported directly to the liver from the small intestine through the portal vein (Fig. 5b). In the liver, a part of the received FAs are oxidized for energy, and a part is modified and packed in lipids of VLDL particles before release back into the bloodstream and consequently delivered to the tissues whereafter LDL is recycled back to the liver, much like mechanisms described above for the mammalian FA metabolism. Ultimately, while all bird tissue FAs may have undergone modification in the liver, some dietary FAs are incorporated in mammalian tissues unmodified (Klasing 1998, Williams & Buck 2010).
Figure 5. Scheme of the uptake of dietary fatty acids (FA) in **a)** mammals and **b)** birds. In mammals, dietary FAs are transported unmodified in triacylglycerols (triglycerides, TAGs) of chylomicrons from the gut to the tissues, where after the chylomicron remnants are transported to the liver. In liver, very low density lipoprotein particles (VLDL) containing dietary/modified FAs, de novo synthetized FAs and cholesterol are synthesized and released into the bloodstream, from which FAs from VLDLs are distributed to tissues. In birds, all absorbed dietary TAGs are transported in portomters to the liver, where the FAs are either oxidized in the β-oxidation, modified, and/or used in the synthesis of VLDLs for distribution to peripheral tissues. LPL, lipoprotein lipase; LDL low density lipoprotein. From Williams & Buck 2010.
1.4.1 Vertical differences in seal blubber

Blubber is a tissue type generally classified as a mammalian trait. Clear compositional vertical differences of blubber FAs are commonly reported for pinnipeds and whales, whereas vertical compositional differences are nearly non-existing in bird superficial adipose tissue. Thus, the vertical stratification of FA in blubber is a unique property of marine mammals and may provide mechanistic information on their physiological adaptation to cold water (Strandberg et al. 2008;2011).

1.5 The Baltic Sea and the studied seals and cormorants

The Baltic Sea is a brackish water area with a surface area of 382 000 km² and is found on the Northern hemisphere, with an ascending salinity gradient from the Gulf of Bothnia in the north to the southern Baltic Sea. Its biotopes consist of shallow bays, archipelago and open pelagic water. The Baltic Sea is connected to the North Sea and the Atlantic by the Kattegat and the straits between Denmark and Sweden. The Baltic Sea receives salt water input through the straits, although the salt water pulses have occurred less frequently since the 1960s and the salinity in the Baltic Sea has continuously declined (Leppäkoski & Olenin 2000, Leppäkoski et al. 2002). Both prey and predator stocks have undergone big changes during the last decades in the Baltic Sea. Multiple factors such as a declining water salinity, increasing eutrophication, heavy lipophilic toxin loads in the 1950s and 60s, current climate change, past excessive hunting of top predators and continuous overfishing of economically valuable fish stocks, have together led to changes in population sizes and species community structure at every trophic level of the Baltic Sea (Bonsdorff et al. 1997a;b, Vuorinen et al. 1998, Wasmund & Uhlig 2003, Österblom et al. 2007).

Since the 1990s, following protective measures that were applied due to a severe decline in population size during the 1960s and 1970s, the Baltic Sea top predator grey seal (Halichoerus grypus) population has been revived. The grey seal is from a global point of view a small-sized pinniped distributed along the shores of the North Atlantic and in the Baltic Sea. After the strong decline of the Baltic grey seal population due to organochlorines and exaggerated hunt, the recently increased population was estimated to reach approximately 30 000 individuals in 2014 (Hårding & Härkönen 1999, Luke 2016).

Another top predator of the Baltic Sea, the great cormorant, returned to the Baltic Sea in the late 1940s after being absent for decades (Ericsson & Carrasquilla 1997, Engström 2001, Bregnalle et al. 2014), and the population has recently expanded and was estimated to exceed 160 000 breeding pairs already in 2010 (Bregnalle et al. 2014) and only the very recent natural predation pressure e.g. from white-tailed eagle may start to limit the population size in some areas (Rusanen 2019). However, the cormorant population present in the Baltic Sea area today consists of two subspecies, the Phalacrocorax carbo carbo and Phalacrocorax carbo sinensis (later Pcc and Pcs, respectively), which exhibit different migration and nesting strategies (Marion & Le Gentil 2006, Frederiksen et al. 2018), and of which Pcs is the subspecies breeding in the Baltic Sea area. The breeding colonies of Pcc are mainly found along the North Atlantic coast of Norway. Like the grey seals, cormorants are strictly piscivorous and may locally cause strain on fish populations.
and economic losses for fisheries in the form of damage to catch and gear. This has created conflict between the top predators and fisheries in many areas (Butler et al. 2011, Varjopuro 2012, Bowen & Lidgard 2013, Rusanen 2014, Friedland et al. 2017, Hansson et al. 2018, Nordberg & Salmi 2019).

When the traditional diet monitoring methods of examining the top predators’ gastrointestinal tract content, faecal scats, regurgitates and pellets, provide detailed snapshot information on the very latest diet (Jobling & Breiby 1986), tissue chemical analyses provide information on long-term foraging behaviour (Bradshaw et al. 2003, Budge et al. 2006). Previously, diet of the Baltic grey seals has been studied by using traditional method (Söderberg 1975, Tormosov & Rezvov 1978, Lundström et al. 2007;2010, Kauhala et al. 2011), and diet studies on Baltic marine birds has not been previously conducted. To be able to more reliably estimate the possible effects of increased grey seal and cormorant predation on fish stocks and the Baltic food webs, already challenged by various anthropogenic factors such as commercial fishing and climate change, accurate knowledge about the foraging habits of these marine top predators is needed. Establishing the use of chemical tissue markers, especially after the tissue sampling protocols are optimized and standardized, could help in receiving reliable long-term information on the diets and feeding habits of the Baltic top predators, as indicated by the grey seal and cormorant studies we conducted.
2. Aims of the study

The present large and growing populations of seal and seabird top predators in the Baltic Sea area call for a need to establish accurate diet monitoring methods in order to mitigate the risen predator-fishery conflicts and meet the requirements of ecosystem-based management initiatives and multi-species ecological assessments. The tissue FA composition as a proxy of diet in food web studies is an established method but the effect of biological factors on the adipose tissue FA profiles are still poorly known. In this thesis project work we aimed to:

1) Study the influence of different factors on the vertical distribution of different FAs that cause the FA profiles at different tissue depth to segregate. Based on these analyses we were hoping to be able to present recommendations on suitable sampling methods for small size pinnipeds. Once the tissue sampling for the FA approach was standardized, we aimed to test for its applicability for assessing diets of different grey seal individuals collected from the Baltic Sea.

2) Evaluate the usefulness of the FA profiles as long-term dietary markers by comparing the results from the FA approach for the Baltic grey seal to those obtained by using tissue stable isotope approach and traditional and DNA-based studies of prey remains in the gastrointestinal tract.

3) Study how well the adipose tissue FA profiles serve in finding spatial and temporal differences in the diets of the two subspecies of great cormorant found in the Baltic Sea area.

4) Compare the applicability of the FA profiling for dietary studies in a seal versus seabird, using as models the Baltic grey seal and great cormorants. We study the ways in which the different physiology and foraging ecology of these animals influence the sensitivity of the FA markers in revealing individual dietary differences.
3. Materials and methods

The research works comprising this thesis were initiated by sampling whole fish, seal blubber (Part I, II) and bird adipose tissue (Part III) from which FA methyl esters were produced and analysed by gas chromatographs coupled with a flame ionization detector (GC-FID) or a mass selective detector (GC-MS) (Parts I-III).

3.1 Sample collection

3.1.1 Grey seal and fish samples (I, II)

Seal and fish samples were collected in 2010-2012 by Swedish, Estonian and Finnish officials from ICES subdivision (SD) areas SD27, SD29, SD30 and SD32 (Fig. 6a; panel 6b presents cormorant sampling sites discussed below). The sampling was in Sweden promoted by the Environmental Protection Agency (www.svetshepa.se) and the Agency for Marine and Water Management (www.havochvatten.se) and carried out by the University of Agricultural Sciences (www.slu.se) and Museum of Natural History (www.nrm.se). In Finland, the sampling was conducted by the Finnish Game and Fisheries Research Institute, currently the Natural Resources Institute (www.luke.fi).

3.1.1.1 Seal samples

For the grey seal individuals, background information such as age (assessed from longitudinal sections of the canine teeth (Hewer 1964) and categorised, 0-4 years = subadults and >5 years = adult), sex, time and area of capture, cause of death (bycaught in fishing gear or shot in open water) and type of fishing gear in case of bycatch were recorded. Blubber samples including skin and muscle were taken from sternum region and frozen at -20 °C until derivatization for FA analysis. When preparing tissue samples for FA analysis, blubber samples were immersed in liquid nitrogen and immediately subsampled across the blubber column 3 mm apart from skin to muscle. A total of n = 108 individuals (Part II) with sufficiently recorded background data were included in our studies, of which n = 30 adult grey male individuals with blubber thicker than 36 mm were studied for factors affecting the vertical distribution of FAs in the blubber (Part I).

3.1.1.2 Fish samples and reference library

From material consisting of a total of n = 433 fish and 26 species, a fish tissue FA (analysed at the University of Helsinki) and SI (analysed at the University of Jyväskylä) library including 11 key fish species from the ICES areas SD27, SD29, SD30 and SD32 (representing different habitats of the Baltic Sea) was composed for these specific prey-predator studies (n(FA) / n(SI) = 233/216). On average 6 individuals/species/SD area were sampled as whole fish samples, and stored in -25 °C for a maximum of six months until homogenization and preparing samples for FA analyses. The storage time has negligible effect on FA composition of these kind of tissues we sampled, as evidenced by Lind et al. (2012;2018). The full fish data were used to establish which
Figure 6. Sampling sites in the Baltic Sea area for the a) seal individuals and fishes included in Parts I and II (as indicated in the figure text boxes by the Roman numbers), and b) cormorant individuals included in Part III.
FAs were the ones responsible for the largest interspecies variation and thus were the most informative for dietary studies since they could cause variation in the FA composition of predator tissues. Thus, the FAs and SIs of the fishes were necessary reference data required to demonstrate the ability of FAs and SIs to distinguish pelagic, coastal predatory and demersal fish. In the reference library, pelagic habitat was represented by Baltic herring, sprat *Sprattus sprattus*, salmon *Salmo salar* and trout *Salmo trutta*, whereas pike perch *Sander lucioperca*, pike *Esox lucius* and perch *Perca fluviatilis* were the characteristic coastal predators foraging in shallow bays. Additionally, common whitefish *Coregonus lavaretus*, roach *Rutilus rutilus* and eelpout *Zoarces viviparus* are known to feed close to the bottom in shallow waters, and represented bottom invertebrate feeders in the reference material. The European eel *Anguilla anguilla* was also included in the reference library to represent a migratory fish species.

### 3.1.2 Great cormorant samples (III)

Great cormorants (n = 94, subspecies confirmed in n = 77) were collected by hunters and officials (authorized by County Administrative Boards in Västernorrland, Kalmar and Blekinge in Sweden and by Miljöbyrån in Åland) in 2013, 2016 and 2017 from the Gulf of Bothnia, the Archipelago Sea, and the north and south of the Baltic Sea Proper (Fig. 5b). The 2013 cormorants were collected in August from the archipelago of Turku, Finland (n = 10, Archipelago Sea). In 2016 and 2017 a total of 29 individuals were collected in Blekinge, southern Sweden (southern Baltic Sea Proper). In 2017, cormorants were collected from Kalmarsund, Sweden (n = 17, northern Baltic Sea Proper), Åland islands (Archipelago Sea), Finland (n = 16) and Sundsvall, Sweden (n = 22, Gulf of Bothnia). Sex of the individuals as well as time and location of collection was recorded. An age estimation based on plumage was made and the studied individuals were categorized as either juveniles or adults (cormorants obtain adult plumage by 3 years of age). Included in the analyses are morphologically subspecies-confirmed individuals (n = 77), with the exception of individuals collected from Kalmarsund, of which only one leg was delivered and thus did not allow for morphological subspecies determination. These individuals were included to improve the representativeness of the data of the Southernmost sampling sites. Whole birds or legs were frozen in -20 °C until sampling. Upon sampling, 10 mm³ subcutaneous adipose tissue was sampled and underwent the lipid transmethylation procedure to yield the derivatives of FAs for gas chromatography.

### 3.2 Grey seal blubber layer determination (I, II)

To determine the grey seal blubber layers and to choose the representative subsamples for the outer, middle and inner blubber layers, we studied the vertical mol% profiles of the five quantitatively most significant FAs present in the blubber (16:1n-7, 18:1n-9, 16:0, 20:5n-3 and 22:6n-3) (Part I, Fig. 6a), and then calculated the numerical gradients (NG) between adjacent subsamples across the blubber column, to assess the blubber depths where the vertical compositional change was the largest, as earlier performed by Strandberg et al. (2008;2011) (Part I, Fig. 6b). Based on the compositional differences verified by statistics between the subsamples taken
from different depth in the blubber column, the outer layer was determined to span an 18 mm layer, which is affected by the ambient temperature (Irving & Hart 1957). The single subsample that represented the outer layer was chosen to be the second outermost subsample (3-6 mm from skin, to avoid the possible influence of connective tissue on the FA profile). The metabolically active inner blubber layer was considered to span approximately 9 mm of blubber from muscle, and the representative subsample for the inner blubber was chosen to be the second innermost subsample (3-6 mm from the muscle, to avoid the possible influence of muscle tissue on the FA profiles). The subsample representing the middle layer is the one found midway between the outer and inner blubber layers (Fig. 7). The depths of the boundaries between the vertical layers in the grey seal are in line with the previously reported boundary depths for ringed seal blubber (Strandberg et al. 2008; 2011).

![Figure 7](image)

**Figure 7.** Schematic picture of grey seal blubber, showing the blubber layers, the subsampling procedure and layer representative subsamples. The outer blubber layer spans the superficial 18 mm of the blubber, while the inner blubber layer spans the innermost 9 mm of the blubber. The middle blubber consists of the blubber tissue between the outer and inner blubber layers. The outer blubber is poikilothermic while the middle and inner blubber layers are thermally stable. Pinja Kettunen, modified from Part I Fig. 1).

### 3.3 Laboratory methods

#### 3.3.1 Preparation of fatty acid methyl esters (FAMEs) (I, II, III)

Adipose tissue FAs were subjected to transmethylation that produced FA methyl esters (FAMEs) as described in e.g. Strandberg et al. (2008). In short, the adipose tissue FAs were heated in 1% methanolic sulphuric acid under nitrogen atmosphere, and hexane was used as co-solvent. Later the FAMES were extracted into hexane, and dried and concentrated for gas chromatography.
3.3.2 Fatty acids included in analysis

For seal and fish studies, the FAs that showed the largest interspecies variation in the fish tissues, the quantitatively important FAs, the mammalian and avian de novo-synthesized FAs, as well as the precursors of essential PUFAs and the PUFAs themselves were included. Trace FAs that would have brought in methodological variation without biologically meaningful information were decluded.

In parts I and II a total of 15 FAs, consisting of the 9 FAs selected based on the largest interspecies variation among the Baltic fish in Part II (14:0, 16:1n-7, 18:1n-9, 18:2n-6, 18:3n-3, 18:4n-3, 20:1n-7, 20:4n-6 and 22:6n-3) as well as the 6 important metabolic substrates and derivatives of them (14:1n-5, 16:0, 18:0, 18:1n-7, 20:5n-3 and 22:5n-6), were analysed. In Part III, all 57 FAs that were reliably detected by GC were included in the analyses. This was a measure of caution, since for the sampling years of the cormorants no prey collection was carried out in the Baltic Sea area, and the birds, if coming from outside the Baltic area, may have FAs not found in the Baltic Sea food web in marked amount. Since such representative prey FA profile library as we had for the grey seals was not available for the cormorants, preselecting FA markers would not have been justified, even if it would have improved the sensitivity of the method in revealing spatial or temporal differences.

3.3.3 Gas-Liquid chromatography (GC) (I, II, III)

The tissue FAME composition was quantified by GC-FID (Shimadzu GC-2010 Plus) and the individual FA structures were identified by GC-MS (Shimadzu GCMS-QP2010 Ultra). For comparability, both equipment manufactured by Shimadzu Scientific Instruments, (Kyoto, Japan), were equipped with Zebron ZB-wax capillary columns (30 m, 0.25 mm ID and film thickness 0.25 µm; Phenomenex, Torrence CA, USA). In short, the FA analysis comprised of injection (AOC-20s autosampler) of the FAMEs into the GC column by AOC-20i autoinjector at 250 °C, and the FAMEs were separated in the capillary column in a temperature-programmed run (180 °C for 8 minutes, then ramped by 3 °C/minute to 210 °C, which was kept for 50 minutes), and finally detected by FID in 280 °C for quantification. When electron impact MS detection was used, the mass spectra including M+ ion of varying intensity and the numerous fragment ions indicative of the chemical structure were recorded for identification.

3.4 Data analysis and statistics

3.4.1 Data-analysis (I, II, III)

The peak areas for individual FAs in the chromatograms produced by GC-FID were defined by manually determining the baseline. Empirical correction coefficients for mass% introduced by Ackman (1992), were used to remove discrepancy due to different burning responses of the carboxyl and other carbons of the chain. Finally, tissue FA compositions were expressed as mol%-profiles. Internal standard FA solution (Supelco 37 Component FAME Mix, Merck KGaA, Darmstadt, Germany) was used to ensure equal burning response for different FAs.

Relative standard deviations (RSD = SD/Mean, also known as coefficient of variation CV) were calculated for each FA for the grey seal and cormorant FA mol%
data (latter shown in Part III) to indicate which FAs showed the largest variability and thus could sustainably show dietary effects. In theory, the *de novo* synthesized FAs would show the smallest RSD values.

### 3.4.2 Multivariate Principal Component Analysis (PCA)

Grey seal, fish and great cormorant FA data were subjected to multivariate Principal Component Analysis (PCA, Sirius 8.5 software, Pattern Recognition Systems, Bergen Norway) for assessment of geographical, seasonal and anatomical compositional differences in the adipose tissue FAs. Prior to analyses, seal and fish FA data were arcsin transformed and FA variables were standardised to reduce a possible dominating effect of major variables (*i.e.* the SD of each FA variable was forced to get the same value of 1), and give all the selected marker FAs equal importance in the analysis (Käkelä et al. 2009). Cormorant FA data was likewise arcsin transformed but subsequently analysed with no other transformation performed on the FA variables, which choice for the data including large number of variables allowed the quantitatively most important components to influence the separations more than the trace components did. The relative positions of the individual samples and variables were plotted in biplots using the first two principal components, explaining the main part of the data variation.

In addition to these descriptive PCA analyses, quantitative analyses were conducted to test the statistical significance of the separations between the sample groups by using Soft Independent Modeling of Class Analogy (SIMCA, Wold & Sjöström 1977, Sirius 8.5 software, Pattern Recognition Systems, Bergen Norway, Dunn & Wold 1980). These tests were pairwise and regarded $P < 0.05$ as significant.

The effect of the choice of the multivariate statistical method was studied by repeating the analyses first made by PCA by using next multidimensional scaling, which is a non-parametric method. It was revealed that the results and their interpretations remained the same when performed either by PCA or non-metric multidimensional scaling (nMDS).
4. Results and Discussion

4.1 Part I: Diet FA composition affects grey seal blubber FA composition

To the eye, blubber is a homogenous tissue but biochemically, it varies in FA composition vertically throughout the column (Best et al. 2003, Guerrero et al. 2016, Guerrero & Rogers 2017). In order to determine the optimal sampling depths of seal blubber, we at first studied the FA compositions in all the vertical 3 mm subsamples from skin to muscle, and prepared full vertical mol% profiles for each FA (Fig. 8).

![Figure 8](image)

Figure 8. Vertical fatty acid profiles (for the 5 main fatty acid components with 3 mm intervals) for 4 individuals showing varying stratification patterns. From Part I ESM 2.

Next, we determined the blubber depths at which the change in composition occurred most rapidly and distinguished between outer, middle and inner blubber layers (Fig. 9) (Part I Fig. 3).
Figure 9. Bidirectional vertical profiles of numerical gradients (NGs) calculated by using the quantitatively most important fatty acids in the blubber of 30 adult male grey seals. In the upper panel, the NGs starting from the outermost blubber layer (0-30 mm), and in the lower panel, the innermost layer (81-90 mm), irrespective of the total blubber thickness of the individuals are presented. The large open circles represent reference subsamples, and the depth where the NGs significantly ($P < 0.05$, Student’s T-test) differed from the reference point is marked with an asterisk. Error bars represent ± SE. From Part I Fig. 3.

We then used the representative subsamples for each layer, subjected the FA data of the representative subsamples from outer, middle and inner layers of 30 adult grey seal males with a blubber thickness > 36 mm to multivariate PCA and plotted all individuals and representative subsamples in a biplot (Fig. 10) (Part I Fig. 7). Pairwise statistical testing showed that while the middle and inner blubber representative subsamples are compositionally more similar, the outer blubber representative subsample has a unique composition in all studied individuals, statistically
significantly different from the middle and inner layer representative subsamples. The FAs driving this separation of the outer representative subsamples from the corresponding representative subsamples of the middle and inner blubber were the MUFAs 14:1n-5, 16:1n-7 and 18:1n-9, all enriched in the outer blubber layer representative subsample. The representative subsamples of the middle and inner blubber are, in turn, enriched in SFAs such as 14:0, 16:0 and 18:0, as well as PUFAs such as 20:5n-3 and 18:4n-3.

**Figure 10.** PCA biplot (using 15 main fatty acids as loadings) of the representative subsamples for the fatty acid composition in outer (blue), middle (brown) and inner (red) blubber of the 30 adult male grey seals marked by the identification code of the individual. The statistical significance of the compositional differences were tested by pairwise SIMCA tests ($P < 0.05$) and reported as an insert in the upper right corner. From Part I Fig. 7.

Grey seal blubber serves as an on-board energy storage as well as an insulating thermoregulatory layer (Liwanag et al. 2012). Outer blubber is obviously subject to more strict endogenous regulation whereas the inner and middle blubber are metabolically active and largely modified by diet. The outer blubber is also exposed to external temperature variations (Irving & Hart 1957) and the enrichment of MUFA
in the outer blubber is possibly partly a consequence of Δ9-desaturase enzyme activity, an adaptation to low temperatures (activity estimation presented later) (Nakamura & Nara 2004). The middle and inner blubber layers, however, are thermoneutral. Furthermore, observable in the dispersion of scores (=individuals) in the biplot, there was less variability in the composition of the outer blubber representative subsample between individuals than in the representative subsamples of the deeper layers (Fig. 10) (Part I Fig. 7).

In addition to the high amount of MUFAs in outer blubber layer, the mol% of different FAs varies (i.e. is stratified) throughout the column depth. We chose five of the most quantitatively or physiologically significant FAs (16:0, 16:1n-7, 18:1n-9, 20:5n-3 and 22:6n-3) and plotted their mol% as a function of the blubber depth in 3mm intervals for the studied adult male grey seal individuals. While the individual blubber profiles, i.e. the stratification patterns, showed great individual variation in the FA composition of the inner and middle blubber, the FA composition was consistently similar in the outer blubber layers of all individuals, which were enriched in MUFAs in all studied individuals. The degree of vertical stratification was especially pronounced for the MUFAs 16:1n-7 and 18:1n-9 along the depth of the blubber column, however with less stratification if the mol% was readily high in the inner blubber (Fig. 8) (Part I Fig. ESM 2).

To study the distribution of the FAs along the blubber depth, we calculated a Stratification Index (SI, Eq. 1) for each studied FA using the outer and middle layer representative subsample mol % and got a numerical index which is positive if the mol% of a certain FA is higher in the outer blubber layer than in the middle layer.

\[
SI = \frac{\text{mol\%}_{\text{outer}} - \text{mol\%}_{\text{middle}}}{\text{mol\%}_{\text{outer}} + \text{mol\%}_{\text{middle}}/2}
\]  

Equation 1.

The SI of five of the studied FAs (16:0, 16:1n-7, 18:1n-9, 20:5n-3 and 22:6n-3; the SI of 16:0 and 16:1n-7 shown in Fig. 11b; c) showed a statistically significant correlation when the values for all individuals were plotted against their respective mol% in the inner blubber. By reading the crossing point of the line of the x-axis we could determine the inner blubber mol% of the FA at which stratification does not occur (Fig. 11) (Part I Fig. 9). The values readable in the scatter plots are consistent with the complete vertical profiles shown in Fig. 8 (Part I, ESM2), where it can be read that e.g. in individual 1624, a value of 20 mol% of 16:1n-7 in the inner, as well as in the outer blubber, would prevent the stratification of this FA towards the outer blubber layers (Fig. 11c). 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.
Figure 11. Stratification index (SI, outer versus middle blubber) for a) ∆9-DI and individual fatty acid b) 16:0 and c) 16:1n-7 mol% of the studied 30 adult male grey seals 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. From Part I Fig. 9.

This led us to the conclusion that a mismatch between the dietary supply of FA (manifested in its proportion in the inner blubber) and its endogenously set demand in the poikilothermal outer blubber drives the vertical FA stratification.
Potential tissue adaptation to low temperatures may be detected as increased FA desaturation. Although not directly measured, the activity of the $\Delta 9$-desaturation enzyme can be estimated, as previously described by Käkelä & Hyväri (1996), by calculating the ratio of the mol% of MUFAs to their precursor SFA. This desaturation index ($\Delta 9$-DI) is commonly used as a proxy for this membrane-bound enzyme activity since it describes the SFAs potentially being converted to desaturated MUFAs by the enzyme. Next, we calculated a SI for $\Delta 9$-desaturation in a similar way to the calculation of the SI for individual FAs (Eq. 1). The $\Delta 9$-DI was calculated using the mol% sum of SFA and MUFA in the outer and middle layers, and then this SI was plotted against the $\Delta 9$-DI of the inner blubber (Fig. 11a) (Part I Fig. 9a). All studied individuals got positive values of $\Delta 9$-SI, i.e. the $\Delta 9$-desaturation of SFAs to their corresponding MUFAs increased across the blubber column from the innermost layers towards the superficial outer layer. Previous studies have shown, as also seen in our results, that the $\Delta 9$-desaturation activity (concluded from the MUFA/SFA-ratio in tissues) was very high in the most superficial layers (Käkelä and Hyväri 1996, Strandberg et al. 2011) but much lower in the deeper layers (Käkelä & Hyväri 1996, Best et al. 2003, Wheatley et al. 2007, Thiemann et al. 2007, Liwanag et al. 2012).

Due to large vertical differences in the relative concentrations of SFAs and MUFAs, we examined the FA $\Delta 9$-desaturation as a factor inducing the vertical FA stratification in the adult male grey seals (n = 30). For this purpose, we used univariate Generalized Linear Models (GLM). In these analyses, we employed SI for $\Delta 9$-DI (outer versus middle blubber) as the dependent variable, and the explanatory variables employed were the $\Delta 9$-DI of inner blubber, thickness of the whole blubber column and seal age. It turned out (Part I Table 2) that the inner blubber $\Delta 9$-DI and seal age significantly influenced the SI for $\Delta 9$-DI, which however was not affected by blubber thickness, which parameter was therefore excluded from further modelling of the vertical FA stratification. The outer blubber $\Delta 9$DI was found to correlate negatively with the age of the seals. In contrast, the outer blubber PUFA contents did not correlate with the age of the individual (Part I Fig. 8). Apparently, the outer blubber $\Delta 9$-DI is not adjusted according to short term thermal conditions, since the season and the water temperatures of the surface water during the collection days of the grey seal males (4−18 °C) had no statistically significant effect on the outer blubber $\Delta 9$-DI (Part I Table 1). Concerning the vertical distribution of individual FAs, conducting the GLM regression analysis indicated that the degree of stratification of an individual FA was largely dependent on the inner blubber proportion of that specific FA. Only the SI of 16:0 and 16:1n-7 were influenced by age (Part I Table 3).

It is noteworthy that PUFAs do not enrich in the outer blubber layer (Part I Fig. 8d) and the mol% of PUFAs is, in fact, almost evenly distributed along the blubber column, suggesting negligible role in adjusting the outer blubber properties. During exposure to low temperature (Liwanag et al. 2012, Radnaeva et al. 2017), the cell membranes and lipid droplets of cytosol need to retain their semi-fluid state, which is achieved by the combined melting point lowering effect of MUFAs and PUFAs. From the point of view of adjusting tissue lipid fluidity, either the increased PUFAs or MUFAs could in theory provide sufficient fluidity (Radnaeva et al. 2017), and thus additional functional or structural requirements or availability from diet determine
the FA composition. Seal diets are rich sources of highly fluid PUFAs and thus outer blubber enrichment of MUFAs instead of PUFAs need to have other structural or metabolic explanations. It should be noted that tissue mechanical strength can be achieved by an enrichment of membrane sphingomyelin (SM) (the ratio of SM to phosphatidylcholine PC), as well as an enrichment of cholesterol (calculated as the ratio of membrane cholesterol to total membrane phospholipids PL) in the outer blubber layers compared to the inner blubber layers. Both high SM and high cholesterol contribute to increased rigidity of the cell membranes of the outer blubber, hence giving mechanical strength for the outer blubber. The acyl chains incorporated into SM are either SFAs or MUFAs and thus result in tight membrane lipid packing increasing the short distance attractive forces between the molecules. Due to compatible molecular shapes and hydrogen bonds, the SM and cholesterol are more compatible with each other (Ramstedt & Slotte 1999, Slotte 2016) than with PC or other PUFA-containing GPLs (Wassal & Stillwell 2009).

In addition to forming of physical protection, blubber also employs a thermoregulatory role, the efficacy of which is determined not only by the thickness of the blubber, but by the maintenance of sufficiently thick outer blubber layer. The specific biochemistry of the outer blubber may support retaining this blubber layer in varying nutritional states (Rosen et al. 2007, Liwanag et al. 2012). The activity of Δ9-desaturase and its main product, 16:1n-7, has been shown to promote lipogenesis in mammalian tissues (Hodson & Fielding 2013). Thus, in pinniped outer blubber this property may allow to maintain this from a thermoregulatory point of view the most important part of the blubber even in a negative energy state. 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 nonselective dietary FA incorporation and thus the middle and inner blubber layers are likely to be the sites that reflect diet better. We therefore assume that while the enrichment of MUFAs in the outer blubber layer is largely set by physicochemical demands such as cell membrane fluidity and tissue mechanical strength (which would be poor with excessively high concentrations of PUFAs in the tissue) the inner blubber criteria of incorporating dietary FAs to TAG molecules in normal mammalian tissue temperatures are less strict. Thus, depending on the dietary supply of FAs, the disparity between the relatively stable outer blubber and the variable inner blubber reflecting the dietary FAs can be large or small. Therefore, the seal blubber vertical stratification can be regarded as a diet dependent phenomenon but equally well also due to genetically controlled composition of outer blubber (Budge et al. 2004, Strandberg et al. 2008, Liwanag et al. 2012).

The inner blubber layer is the metabolic interphase and dietary FAs are first incorporated in this layer (Budge et al. 2004, Guerrero et al. 2016). To test for the ability of the different blubber layers to reveal individual diet, we conducted a series of tests where the outer, middle and inner blubber representative subsamples were subjected to PCA. We found that the sensitivity of the method to indicate diet diminishes towards the outer blubber (Fig. 12) (Part I Fig. 10). This is observable by the ability of PCA analysis to maintain the individual groups that initially clustered into clear groups (T=Trawl, R=Red, G=Green, B=Blue) when the mol% values from the
inner blubber subsamples were used as loadings. Using the grouping made by PCA, we subjected the middle and outer blubber representative subsamples to the same analysis and found a reduction in cluster formation, observable by less statistically significant differences between groups when analysed by pairwise comparisons (SIMCA). While the pairwise comparisons between the groups were all significant when using the inner blubber representative subsample data, certain comparisons became non-significant when using the representative subsample data of the middle or outer blubber. To gain further understanding of the usefulness of blubber in describing seal dietary habits and the sensitivity of FA analysis as the method of choice, we conducted comparisons also by using mean values of all subsamples throughout the whole blubber column, or by omitting either the outer six subsamples (18 mm) or the inner three subsamples (9 mm) from the calculations (Fig. 12). Using the mean values did not cause large loss of information, but the comparisons made after omitting the representative samples of the outer layers were statistically significant for all the comparisons between the different groups of individuals. When omitting the inner blubber layer subsamples, several pairwise comparisons failed to reach statistical significance. If the whole blubber column was used, i.e. the full depth mean, 5/6 of the comparisons were statistically significant.

It is known that dietary lipids not immediately needed for recent energy metabolism, are incorporated into adipose tissue from the bloodstream on a minute time scale after ingestion (Cooper et al. 2005, Frayn et al. 2006). This suggests that the new dietary FA signal in the metabolically active inner layers may be observable already shortly after prey ingestion. The enrichment of dietary PUFAs into the inner and also middle blubber layers further underlines the assumption of higher metabolic activity in the inner and middle blubber layers compared to the outer blubber layers (Iverson 2008, Strandberg et al. 2008; 2011). This view was also supported by studies on ringed seals from Lake Saimaa where the closest compositional similarity with the potential prey fish FA composition was found in the innermost blubber. Simultaneously, large interspecific differences in inner blubber FA composition were found between the ringed seal individuals from Lake Saimaa and the Arctic Ocean, whereas the outer blubber of the lacustrine and marine subspecies had relatively similar composition (Strandberg et al. 2008; 2011).

Since the inner and middle blubber layers are metabolically active, diet merely affects inner and middle blubber composition, while the composition of outer blubber is largely set by requirements or factors other than diet (Budge et al. 2004, Strandberg et al. 2008, Guerrero et al. 2016, Bourque et al. 2018). Previously, diet assessments have been conducted by using the whole blubber column (Bradshaw et al. 2003) or a rough inner vs outer blubber categorization in which the blubber column is divided into two halves (Käkelä & Hyvärinen 1993, Liwanag et al. 2012, Guerrero & Rogers 2017), and while it gives a useful estimate on diet composition, more accurate information is obtained if the outer blubber is omitted from the analysis.
Figure 12. PCA results (15 main fatty acids as loadings) of the blubber fatty acid composition of 30 adult male grey seals (individuals by their identification codes) by using data from different sampling procedures. The PCA biplots of a) inner, b) middle and c) outer blubber representative subsamples are shown first. Next the PCA biplots using the d) mean values combining full inner and middle blubber layers (without outer layer), e) mean values of the whole blubber column, and f) mean values combining the subsamples through the middle and outer blubber (without inner layer subsamples). Results of the pairwise SIMCA comparisons as inserts ($P < 0.05$; NS = nonsignificant). Groups (Part I Table 1): T = Trawl (bycaught in trawl), B = Blue, R = Red, G = Green, and the individuals coded grey represent the “remaining” group.
Next, the addressed time window determines whether the inner blubber or the middle blubber integrating dietary FAs over a longer period should be examined. The innermost blubber FA profiles would be highly interesting when studying the recent diets of seals but if the animal is fasting, the innermost blubber FAs are hydrolysed and modified in too large extent to be used as a reliable source of information in diet monitoring. It should however be realized that the time window of the diet, which is addressed will be different. In addition to findings made by e.g. Strandberg et al. (2008), our work on Baltic grey seals strongly suggests that the inner blubber likely represents the diet of weeks, and when also the middle blubber is included the time window is elongated to many months.

Thus, regarding the possible sampling procedures, the most sensitive sampling site to indicate diet is likely the inner blubber. An additional finding also speaking for omitting the outer blubber layer from dietary studies is the interesting discrepancy in the age-dependent MUFA enrichment in outer blubber found between our grey seal data and earlier data on ringed seals captured in 1992 and 2000 from the Baltic Sea and from Svalbard (Käkelä & Hyvärinen 1996, Strandberg et al. 2008). In ringed seal, we saw a trend of the outer blubber Δ9-DI increasing with age, whereas the grey seal showed an opposite trend in the same comparison, namely a decreasing outer blubber Δ9-DI with age (Part I, Fig. 8). The result suggests that there may be species-specific age dependent variability in the proportions of MUFAs and SFAs in the outer blubber, which in dietary studies could compromise the results. Another noteworthy factor to consider in diet studies is the variation of blubber thickness. The influence of the outer blubber with a fixed thickness of about 18 mm on the FA composition would be larger in an individual with overall thin blubber, if the whole blubber column was used. Among seal individuals with varying blubber thickness, this would bring in variability not related to diet.

4.2 Part II: Fatty acid analysis reveals individual long-term foraging habits whereas stable isotopes may reveal migration in Baltic grey seal

To be able to produce valid dietary studies of free-ranging mammals, information on the FA composition of all relevant dietary items should be at hand. We conducted analyses of the whole fish tissue FAs and SIs from 11 key species fish captured in the Baltic Sea from SD27, SD29, SD30 and SD32 (see Fig. 6a for collection sites), representing pelagic, demersal and coastal foraging habitats and species, as well as one migrating fish species (Part II Fig. 2). We found that pelagic fish species (herring, sprat, salmon, trout) were rich in C18 PUFAs, the coastal predatory fish species (pikeperch, pike, perch) were rich in C20-22 PUFAs, such as 20:4n-6 and 22:6n-3, and the demersal species (common whitefish, roach, eelpout) were rich in MUFAs, such as 20:1n-7 and 16:1n-7. The migratory fish species, the European eel, was rich in MUFAs and 14:0. In the corresponding comparisons of tissue element stable isotopes (SIs), the separation of the fish species according to representative habitat was poorer, but on the other hand, tissue SIs, more specifically δ34S, managed to separate the carnivorous pelagic fish species, i.e. the salmonids, from the planktivorous pelagic fish species, i.e. herring and sprat. The coastal predators showed high values
for $\delta^{15}N$ and $\delta^{13}C$, whereas the carnivorous pelagic species showed a high value for $\delta^{34}S$.

Commonly, marine environments have higher values for $\delta^{15}N$ and $\delta^{13}C$ than freshwater environments do, while high values for $\delta^{34}S$ are observed in freshwater biomes (Peterson & Fry 1987, Hobson 1999, Connolly et al. 2004). Although not used as a biomarker in the Baltic Sea area before, the high values for $\delta^{34}S$ obtained in pelagic predatory salmonids in this study, may stem from their life history containing early years spent in fresh water habitat. As $\delta^{15}N$ and delipidated $\delta^{13}C$ represent dietary proteins (Elliott & Elliott 2016), tissue FAs and SIs are proxies of dietary lipids and proteins, respectively, and are thus complementary in dietary studies.

To test the applicability of different tissue chemical markers for dietary studies in the Baltic sea, we subjected the blubber FA and muscle and liver SI data of a total of $n=108$ seal individuals from areas SD27, SD29, SD30 and SD32 to PCA. Due to large individual compositional variation in the blubber FAs, this marker failed to cluster seal individuals into specific groups according to any studied parameter such as age, sex, SD area of capture and fishing gear type (in case of bycaught individuals or other collection method) (Fig. 13a) (Part II Fig. 3). This does not mean that the method would not work. Rather, it means that the seals are largely opportunistic predators and have very individual diets and foraging habitats. Blubber FAs thus reveal each studied individual’s FA composition and gives information on dietary habits over a long period of time. We also tested the ability of SIs of seal muscle and liver to reveal individual dietary preferences. We found that whereas muscle SIs were not able to group the studied individuals according to dietary specialization, liver SIs grouped the studied individuals according to capture site (SD areas), except for a few (7) individuals, captured in the western SD areas of the Baltic (SD27 and western SD30) (Fig. 13b) (Part II Fig. 3), but which had SI markers that were similar to those of the individuals captured in the eastern part of the Baltic Sea (SD29, eastern SD30 and SD32). In the absence of other explaining factors in these individuals (such as age, sex, or even differing FA composition from their peers in the western Baltic), we suggest a recent migration westward as the probable explanation to the differing spatial liver SI markers. Oksanen et al. (2014) have shown that grey seals can span areas of 60x120km$^2$ during a couple of months. Given that the SI turnover rate in liver is estimated to be 6.4 days (discussed below), we assume that the reason for this grouping is migration from the Finnish coast to the Swedish coast over a few weeks preceding capture in SD27 and western SD30 (Fig. 13b) (Part II Fig. 3).

However, it is noteworthy to take into consideration that as for tissue chemical markers, FAs and SIs reveal different kinds of dietary information from different time windows. Since dietary FAs are not degraded in digestion and absorption, they are incorporated into adipose tissue in a predictable manner and function as a reliable proxy of dietary FAs. Tissue SIs, however, reveal the geographical origin of the diet and give also information about different dietary components and their average trophic levels (Hobson 1999, Newsome et al. 2010). As previously suggested in Part I, the inner blubber is likely the metabolically most active layer, assumed to reflect dietary changes in a week scale, as seen from its large FA profile variability and sensitivity to dietary changes (Strandberg et al. 2008;
While the outer blubber composition remains relatively taken intact, the middle blubber also carries ecological dietary information in the scale of months. Regarding liver and muscle SIs, the dietary information that can be extracted from the tissues represent two different time window categories (Rubenstein & Hobson 2004, Dalerum & Angerbjörn 2005). According to previous mammalian studies, the turnover of SIs in liver is faster than in muscle (Tiezen et al. 1983, Hildebrand et al. 1996, Kurle & Worthy 2002, Phillips & Eldridge 2006). In liver, the carbon half-life was reported to be 6.4 days while the half-life in muscle was reported to be 27.6 days. Even older dietary signals from a time period of roughly 2-3 times that of the isotopic half-life may be detected in tissues (Kurle & Worthy 2002, Phillips & Eldridge 2006). The overall blubber FA turnover rate has in the case of another pinniped, the harbor seal (Phoca vitulina), been estimated to be 2-3 months (Nordström et al. 2008), which can be used as a valid estimate for the middle blubber FA turnover rate. Hence it is valid to assess the innermost blubber FA turnover rate to be much shorter. Thus, likely the inner blubber FAs and liver SIs ($\delta^{13}$C, $\delta^{15}$N and $\delta^{34}$S values) reveal dietary information of a few (2-3) weeks prior to tissue sampling, while middle blubber FAs and muscle SIs reveal dietary information of a few months.

When handling a smaller dataset of well-characterized seal individuals (n = 11 adult male grey seals), with precisely known and recorded information on the
area of catching and type of fishing gear they were found in, individual dietary preferences and habits were clearly observed as a grouping of the studied individuals in PCA according to the fishing gear type they were bycaught in. The blubber of the seal individuals in each group had the same typical FAs characteristic of the prey fish generally caught with that specific fishing gear. This pattern was observable both in mid- and long-term FAs from inner and middle blubber, respectively, and the same trend was observable in the SI proxies with corresponding time windows, i.e. liver and muscle SIs (Fig. 14) (Part II Fig. 4). Again, the same SI profiles typical for the prey fish species that were bycaught by the same gears, were visible in the tissues of the seals. For confirmation, we combined the mid- and long-term tissue FAs and SIs and rerun the PCA using as loadings these combined sets of variables. These analyses with combined FAs and SIs variables gave the same separations that were observed when the FA and SI variables were separately used as loadings to PCA (Fig. 14).

Our data on the set of 11 adult male grey seals (habitat recorded and well-characterized by sampling site and gear type) suggested that the tissue chemical markers, FAs and SIs are both useful proxies for monitoring of diet type and origin on an individual level. Blubber FAs failed to distinguish any large dietary groups of numerous individuals (Fig. 13a), which was likely due to the fact that such homogenous dietary groups did not exist but the Baltic grey seals had very individual diets and foraging habits. Once these are confirmed, the seal individuals that are known to have the very same dietary history indeed group together in PCA and have similar blubber FA profiles. Tissue SIs were a good proxy for geographical origin of diet and grouped the studied grey seal individuals according to sampling site, but only when mid-term dietary SI markers, i.e. liver SIs were used. Long-term dietary SI markers from muscle tissue failed to group individuals according to geographical parameters, but may still tell about the average long-term diet on an individual level.
Figure 14. PCA results of 11 adult male grey seal individuals (with precisely recorded backgrounds) indicated by their identification codes and when a) inner blubber FA, b) middle blubber FA, c) liver SI, d) muscle SI, e) combined inner blubber FA and liver SI data, and f) combined middle blubber FA and muscle SI data were used as loadings. Pairwise SIMCA comparisons ($P < 0.05$) are shown in the upper-left corner of the plots (no statistical significance for panel C). T = bycaught in trawl, S = bycaught in surface fyke, B = bycaught in bottom fyke.
4.3 Part III: Adipose tissue fatty acid analysis reveals spatial and temporal differences in Baltic cormorant foraging habits

To test the usefulness of tissue FA analysis in revealing spatial and temporal dietary habits of a marine avian top predator, we analysed the FA composition of subcutaneous adipose tissue from the knee of two cormorant subspecies *Pcs* and *Pcc*, the individuals of which were collected at the Baltic Sea (see Fig. 6b for collection sites). As opposed to the full grey seal data, the full cormorant data we had did reveal

![Figure 15](image.png)

**Figure 15.** Indicative fatty acid ratios (mean + SD) in the adipose tissue of cormorants collected from Sundsvall (S), Turku archipelago (T), Åland Islands (A) and Blekinge (B). The months and number of individuals for each collection are marked under the bars. **a)** Ratio of C18 PUFA/PUFA\(_\text{tot}\) indicating fresh water or brackish water pelagic fish in diet **b)** ratio C20-22 MUFA/MUFA\(_\text{tot}\) indicating ocean zooplankton feeding fishes in diet. The values are molar ratios, and the bars with no common letter differed at \(P < 0.05\) (Kruskall-Wallis ANOVA followed by Kolmogorov–Smirnov test, \(P < 0.05\)). Details are found in Part III, Materials and Methods. From Part III Fig. 9.
clear grouping of individuals based on the adipose tissue FA composition. When the ratio of C18 PUFAs to total PUFAs (PUFA_{tot}) (usually high in freshwater fish) in adipose tissue were calculated and compared, an increasing trend towards North was found (Fig. 15) (Part III Fig. 9), and the C20-22 MUFAs (usually high in oceanic fish) showed a decreasing trend towards North (with the exception of Sundsvall June 2017 individuals discussed further below). When FA data of all studied subspecies-confirmed individuals (n = 77) from all studied collection sites were subjected to multivariate PCA and plotted in a biplot, we observed both geographical and temporal segregation of the studied individuals (Fig. 16) (Part III Fig. 3).

![Figure 16. PCA a) scores and b) loadings plots of adipose tissue fatty acid profiles of 77 cormorant individuals collected from four locations in the Baltic Sea area. Pairwise SIMCA (P < 0.05) as upper panel insert. Sample key for cormorant individuals: [location letter][individual number][subspecies, s or c][sex, m or f][age, a or j]; S = Sundsvall, T = Turku, A = Åland Islands, B = Blekinge, Bw = Blekinge winter 2016, s = Phalacrocorax carbo sinensis, c = P. c. carbo, m = male, f = female, a = adult, j = juvenile. NS = nonsignificant.](image)

The statistically most significant spatial differences in cormorant knee adipose tissue FA composition was found between the northernmost sampling site, Sundsvall, and the southern sampling points Turku, Åland islands and Blekinge (Fig. 16) (Part III Fig. 3). In this comparison, the FAs mainly responsible for the grouping and data variation were the oceanic markers 20:1n-9 and 22:1n-11 found with high mol% values in Sundsvall individuals collected during June, as well as the freshwater marker 16:1n-7, found with high mol% values in Sundsvall individuals collected in August and September (Fig. 17) (Part III Fig. 7). Furthermore, adipose tissue of individuals collected at the southernmost sampling site Blekinge in January 2016 contained relatively high percentages of 20:4n-6, 20:1n-7, 20:1n-11 and SFAs 16:0 and 18:0 (Fig. 16).
Figure 17. PCA a) scores and b) loadings plots of adipose tissue fatty acid profiles of altogether 45 cormorant individuals, which were collected in autumn (Aug, Sep and Oct) from Sundsvall (n = 13), Åland Islands (n = 16), Turku (n = 10) and Blekinge (n = 6). Pairwise SIMCA (P < 0.05) as upper panel insert. Sample key for cormorant individuals: [location letter][individual number][subspecies, s or c][sex, m or f][age, a or j]; S = Sundsvall, T = Turku, A = Åland Islands, B = Blekinge, Bw = Blekinge winter 2016, s = Phalacrocorax carbo sinensis, c = P. c. carbo, m = male, f = female, a = adult, j = juvenile. NS = nonsignificant.

The larger Atlantic ssp of the great cormorant, i.e. Pcc, has previously not been thought to nest in the Baltic Sea area and was assumed to be present in the area only during migration and winter season (Rusanen 2014). Our data, collected in January, March, April, June, August, September, November and October, however, suggests that Pcc individuals are present in the Baltic area during all seasons (Part III Fig. 2b), though no confirmation on nesting is available. From a distance, the subspecies are hard to firmly distinguish morphologically, and we also did not find that adipose tissue FAs would have revealed compositional differences that would be ssp-specific or would have revealed different dietary preferences for the two subspecies (Part III, Fig. 2a).

However, the ability of FA analysis to reveal differences in dietary habits of marine predatory birds on an individual level was clearly shown with the help of the 9 individuals collected from Sundsvall in June. Only 10 days prior to the collection time of the 9 mentioned cormorant individuals, salmonoid smolts were released to the same river from a nearby hatchery. Cormorant predation on the smolts and consequent loss of released smolts have been suggested (Hansson et al. 2018). The oceanic MUFA marker 22:1n-11 was the main FA variable driving the separation of these 9 individuals sampled in June in Sundsvall from the cormorants collected from this or any of the other locations later (Fig. 16) (Part III Fig. 4a; b). In addition, the adipose tissue 18:2n-6 of these individuals was exceptionally high. It is estimated that cormorant spring migration towards north proceeds along the coast line and takes place in April (Rusanen et al. 2014, Toivanen et al. 2014) and thus, the June Pcs individuals with the oceanic LCMUFAs in their adipose tissue were likely Baltic individuals that displayed a signal originating from the fish feed used at the hatchery to raise the salmonids. This fish feed mostly contained high amounts of North
Atlantic and South American fish flour from zooplanktivorous fish species (blue whiting *Micromesistius poutassou*, sardine *Sardina pilchardus* and anchoveta *Engraulis ringens*), while flours from Baltic fish species (herring and sprat) made up only 10% of the fish in the flour feed (BioMar 2018). Moreover, the fish feed was supplemented with fish oils of oceanic origin and soybean and palm oils. Oceanic fish species are characterised by the C20-22 MUFAs and the most common PUFA in soy and palm oil is 18:2n-6 (Tocher et al. 2004, Käkelä et al. 2005, Brown & Hart 2010).

In the light of the information about the freeing of smolts from the hatchery, and about the FA composition of the fish feed used, the peculiar FA composition of the 9 individuals that bring the most variation to the FA profile data, should be regarded as a dietary signal from the hatchery fish, not a pre-migratory dietary signal from the North Atlantic. The turnover rate in the avian superficial adipose tissue is estimated to be about 1-2 weeks (Williams et al. 2009, Käkelä et al. 2010, Wang et al. 2010) and the ocean fish-plant oil signal C20-22 plus the high 18:2n-6 in the individuals captured in June would be observable if they had been using hatchery smolts as frequent prey during the last 10 days.

The tissue FAs indicating differences among the cormorant individuals were studied by calculating the RSD-values for FA variables exceeding a mean mol% value of 0.2. The FA variables with the highest RSD values were 22:1n-11, C20 MUFAs, 16:2n-4, and 22:2 NMID (Fig. 18). The LCMUFAs and PUFAs in general had high RSD values. Interestingly, the FA variables causing the largest variation to the data remained the same even when the smolt-feeding individuals were included, with the slight changes that 20:1n-9 became more important as a variability-causing FA, and the importance of 22:1n-11 became diminished but remained still among the variables with the largest RSD-value. Thus, regardless of the non-disputable role in data variability that was brought in by the smolt-feeding, the FA variable that got the highest RSD values in the cormorants sampled in the Baltic Sea, was still the oceanic LCMUFA 22:1n-11. The dietary 22:1n-11 signal is more persistent than the other FA signals since the 22:1n-11 is not hydrolysed from tissue lipids by lipases as the other FAs (Raclot 2003, Käkelä et al. 2009), which likely gives the 22:1n-11 slow turnover rates.

The fall migration of cormorants is estimated to take place in July-November (Fransson et al. 2001), which leads us to consider that individuals collected in January-March, possibly even the individuals collected in April in Blekinge, could be overwintering individuals irrespective of the subspecies. Since spring migration following coastlines occurs in April (Rusanen et al. 2011, Toivanen et al. 2014), individuals collected in June are likely nesting in the area. Since no absolute time points for migration can be established, individuals occurring in the Baltic Sea area in August, September and October are possibly both nesting individuals, and individuals already commencing their fall migration.

In addition to providing information on spatial dietary differences, tissue FA analysis is also useful in detecting temporal dietary variation, clearly detected in the seasonal comparisons (among the individuals collected from January to August) of subcutaneous adipose tissue FA composition of cormorants captured in the southernmost sampling site, Blekinge. The only pairwise comparison that reached statistical significance was the one between January and June+August individuals,
with the 18:1n-9 enriched in January individuals, and the PUFAs 20:5n-3, 22:5n-3 and 22:6n-3 enriched in the June+August individuals, respectively. In two of the June+August individuals, the LCMUFAs 22:1n-11, 20:1n-11 and 20:1n-9 were enriched (Part III Fig. 5).

4.4 Fatty acid profile variability as population level measure of opportunistic feeding

The variability in tissue FA profiles are valuable not only when studying the diets of individuals but also when characterizing and comparing foraging ecology of populations. The degree of variability of tissue FA profiles (measured e.g. by the RSD values for FAs) is dependent on the occurrence of different prey with different FA composition and their availability for the predators. Thus, the FA profile variability is not necessarily telling whether the species is a specialist or generalist feeder but instead it tells how opportunistic feeder the species is in its environment. Thus, there is promise to develop an “opportunistic feeder index”, which would be useful in comparing foraging habits of aquatic predator species inhabiting the same foraging area. However, in this thesis project, we did not yet develop such opportunistic feeder index since refining and optimizing the parameters for the calculation needs additional research. The choices regarding the parameters, such as the number and threshold concentrations of the FA variables included, the number of individuals required, and the spatial and temporal representativeness of the samples collected from the area, still need to be fixed. Plasma FA RSD values were previously compared between a few North Sea seabirds (Käkelä et al. 2005), which distinguished between specialists and generalist species, but similar comparisons between the seabird species of the Baltic Sea have not yet been performed.

In this study, we were able to compare the differences in the adipose tissue FA profiles of the adult grey seals and the adult cormorants collected in the Baltic Sea area. When calculating and comparing the RSDs of FA variables with a mean > 0.2 mol% for the adult cormorant and adult seal individuals (n = 56/n = 51), we saw that the five FA variables causing the most variability in the seal and cormorant data were C20-22 MUFAs as well as C16 PUFAs. For the adult cormorants (both Pcs and Pcc), MUFAs such as 22:1n-11, 20:1n-11, 20:1n-9 and the PUFAs 22:2 NMID and 16:2n-4 had the highest RSD values, while in the data of adult seals the highest RSD values were found for the MUFAs 22:1n-11, 22:1n-9 and 22:1n-7, and several PUFAs, the 22:4n-3 receiving the highest RSD values (Fig. 18). Considering that the adult grey seals are strictly local to the Baltic Sea but cormorants are able to fly long distances, and thus feed also in other areas than brackish water habitat, the similarities regarding the C20-22 MUFAs that gained the high RSD values are slightly surprising, especially since these FAs are commonly considered as oceanic markers. On the other hand, the fact that 22:2 NMID had high RSD values in the cormorant data still suggests an oceanic origin of the diet, since 22:2 NMID likely originates from oceanic molluscs. This assumption is strengthened by the fact that also 16:2n-4 gained a high RSD value, since 22:1n-11, 22:2 NMID and 16:2n-4 origin from oceanic plankton-filtering molluscs (Irazú et al. 1984, Silina & Zhukova 2009, Lazzara et al. 2012), which are rare dietary items in the Baltic Sea area. The 20:1n-11 LCMUFA which was among
the FA variables with the highest RSD value are likely the shortening products of 22:1n-11 (Käkelä et al. 2009) and its high RSD value in the cormorant data is therefore accounted for. Regarding the grey seals, the high values of C22 LCMUFAs is likely a signal of the degree of rarity they have as dietary markers in the Baltic Sea area and high RSD values of these is likely a consequence of their enrichment in the blubber of a few studied individuals. The dietary origin of the varying but quantitatively small PUFA 22:4n-3 is not known.

Our findings regarding RSD values strongly suggest that an “opportunistic feeder index” should be added as a parameter in dietary studies, to describe the level of variety in prey selection and whether it translates into data on population level. Along with improved and therefore more sensitive sampling protocols and results, the biomonitoring studies on free-ranging marine mammals can further increase its applicability as a tool in diet assessment.

Figure 18. The RSD (SD/mean) values for the studied tissue FAs with a mean value > 0.2 mol% in adult a) cormorants Phalacrocorax carbo sinensis (Pcs, n = 36) and Phalacrocorax carbo carbo (Pcc, n = 20) and b) grey seals Halichoerus grypus (n = 51).
5. Conclusions and future perspectives

When using tissue FA profiling in dietary studies, it is important to recognise the possible physiological or environmental factors that might affect the usefulness of FA signature as a proxy of diet and food web structure. The use of FAs in the monitoring of aquatic food webs is an established method due to the good information-carrying capacity characteristic for FAs, which is a consequence of their assimilation into tissues largely with unmodified structures. Also, the varying ability of different phyla to synthesize and modify certain FAs allows to identify specific dietary items, which adds to the usefulness of these biomolecules as markers for dietary links between aquatic organisms.

However, to further improve the usefulness of the diet assessment methods utilizing tissue FA profiles, a uniform sampling protocol for sampling of pinniped blubber should be established. Due to the physiology and biochemistry of blubber, the strongest recent dietary FA signal is acquired from the inner blubber layer (represented by the second most inner subsample in this study). Using the innermost blubber requires that the individual has not been fasting which would modify the FA composition of the innermost blubber. Although the dietary information is not completely lost when using the whole homogenized blubber column, including the endogenously regulated FAs of the outer blubber layer, notably more accurate dietary signals are obtained from the inner and middle blubber layers. To gain globally comparable dietary proxy data, we advise to strive to sample innermost or middle layer depending on the addressed time window of feeding, but to systematically rule out the outer, thermoregulatory layer due to its endogenously regulated FA composition adapted to cold water.

The tissue FA signature is a useful proxy of diet on an individual level as well as on population level in aquatic mammalian and avian top predators in the Baltic Sea area. Whereas other methods investigating prey remains in digestive tracts, faecal scats, pellets or regurgitates do provide information on most recent diet of a few days, tissue FAs reveal utilised prey type from a period of weeks to months. Certain FAs can be used as specific characteristic markers of certain prey types and habitats, and individual variation in the occurrence of these markers can be traced back to their trophic level and dietary source. Whereas tissue FA analysis is effective in giving an average view of the feeding ecology of a free-ranging animal, it does not give information on prey items at a species level. However, analysis of tissue FA signature in inner and middle blubber does reveal mid- or long-term foraging habits of pinnipeds, and thus information on their frequent prey type.

In the avian phyla with faster tissue turnover rate, the method was shown to be effective also in revealing dietary habits of a shorter time windows, and perhaps also sudden opportunistic utilization of hatchery reared fish. We emphasize the role of seabirds as wide-range samplers of the aquatic food webs, and their ability to discover local fluctuations in prey availability and furthermore their potential to provide information of food web changes even with a time resolution of a couple of weeks. Compared to pinnipeds, the adipose tissue FA analysis method when applied to seabirds is temporally more sensitive due to their faster tissue lipid and FA turnover. This likely adds accuracy to the spatial analyses as previous tissue FA
markers are continuously replaced by new markers mirroring contemporary prey with only a 1–2-week delay. In addition, the RSD-values of the FA variables could be used as a measure of opportunism that the predator’s population employs in its foraging habitat.
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Due to the raging pandemic shaking the world and our existence, certain academic traditions are not present in this defence. To avoid excess hugging in large assemblies, the karonkka, in its traditional sense, will not be held in honor of the opponent. For this reason, I would like to take the chance to thank professor emeritus Esa Hohtola for accepting the task of being my opponent. Thank you for your time and effort. I look forward to our upcoming discussion!

During these exceptional times that we are living right now and with most of you following the dissertation from the safety of social isolation: I urge you to wash your
hands, take a glass of bubbly in both hands, activate your bilateral mirror cells and perform a *skålus singularis* in celebration at the end of the defence.

Skål and thank you all!

Malin Tverin
In social isolation and quarantine
May 2020

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FACTORS AFFECTING THE FATTY ACID PROFILE OF ADIPOSE TISSUES USED TO ASSESS INDIVIDUAL DIETARY HISTORY OF GREY SEALS AND GREAT CORMORANTS IN THE BALTIC SEA

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