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The dietary and thermoregulatory role of blubber as revealed by fatty acids

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The dietary and thermoregulatory role of blubber as revealed by fatty acids

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A thesis in fulfilment of the requirements for the degree of

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Abstract: Blubber was a crucial adaptation for mammals living in water. Blubber serves as an energy reservoir, where surplus energy is deposited in the form of fatty acids (FAs). Most FAs are obtained from an animal's diet (dietary FAs), thus a predator's FA signature has the potential to provide dietary information. However, some FAs in the predator may be synthesised or modified intrinsically to fulfil physiological demands (non-dietary FAs), which complicates dietary studies. The aim of this thesis was to understand how FAs signatures relate to the dietary and thermoregulatory roles of blubber. I analysed FAs of leopard and crabeater seals to determine how FAs vary across the blubber depth and how this variability influenced dietary interpretations.

I found that blubber is not uniform; where some FAs are abundant in the outer (superficial) layer others are dominant in the inner (deepest) layer. This suggests that the inner layer has a dietary role whereas the outer layer has a more structural role. The FA signatures from the predator's inner layer resembled more closely those of their prey than the FAs in the outer layer. Trophic predictions were clearer when using only the dietary FAs rather than all FAs; this indicates that there are other factors influencing the metabolism of the non-dietary FAs. To examine if a mammal's thermoregulatory requirements impact the shifts in FAs, I conducted a meta-analysis including 48 mammals from terrestrial, semi-aquatic, and fully-aquatic environments. I found that the FAs of aquatic mammals are more highly desaturated than those of terrestrial mammals. Higher desaturation helps reduce heat loss and ensures that blubber remains flexible in cold environments. FA desaturation is correlated with latitude and fur density in semi-aquatic mammals. Thus, they increase FA desaturation when living in colder habitats and when they have sparser fur. I compared the FAs of three sympatric Antarctic seals and found that FA desaturation changes as seals grow, which suggests that the thermal efficiency of blubber develops with age. In order to obtain better results in dietary studies, the effect of thermal habitat, fur density and age, on the metabolism of FAs must be considered.

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A mi madre, Teresa, por su amor incondicional

y por creer en mí desde el día uno

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Preface

Some of the chapters of my thesis have been published, or are *in press*, elsewhere:

Chapter 2 has been previously published as:

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AIG and TLR conceived the study. JN, MEM and JM collected the samples. KZ conducted the laboratory analyses. AIG and TLR wrote the paper.

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AIG and TLR conceived the study. AIG collected the samples and conducted laboratory analyses. AIG and TLR wrote the paper.

General abstract

Blubber was a crucial adaptation for mammals living in water. Blubber serves as an energy reservoir, where surplus energy is deposited in the form of fatty acids (FAs). Most FAs are obtained from an animal's diet (dietary FAs), thus a predator's FA signature has the potential to provide dietary information. However, some FAs in the predator may be synthesised or modified intrinsically to fulfil physiological demands (non-dietary FAs), which complicates dietary studies. The aim of this thesis was to understand how FAs signatures relate to the dietary and thermoregulatory roles of blubber. I analysed FAs of leopard and crabeater seals to determine how FAs vary across the blubber depth and how this variability influenced dietary interpretations.

I found that blubber is not uniform; where some FAs are abundant in the outer (superficial) layer others are dominant in the inner (deepest) layer. This suggests that the inner layer has a dietary role whereas the outer layer has a more structural role. The FA signatures from the predator's inner layer resembled more closely those of their prey than the FAs in the outer layer. Trophic predictions were clearer when using only the dietary FAs rather than all FAs; this indicates that there are other factors influencing the metabolism of the non-dietary FAs. To examine if a mammal's thermoregulatory requirements impact the shifts in FAs, I conducted a meta-analysis including 48 mammals from terrestrial, semi-aquatic, and fully-aquatic environments. I found that the FAs of aquatic mammals are more highly desaturated than those of terrestrial mammals. Higher desaturation helps reduce heat loss and ensures that blubber remains flexible in cold environments. FA desaturation is correlated with latitude and fur density in semi-aquatic mammals. Thus, they increase FA desaturation when living in colder habitats and when they have sparser fur. I compared the FAs of three sympatric Antarctic seals and found that FA desaturation changes as seals grow, which suggests that the thermal

efficiency of blubber develops with age. In order to obtain better results in dietary studies, the effect of thermal habitat, fur density and age, on the metabolism of FAs must be considered.

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CHAPTER 1

General Introduction

1.1 Blubber as an adaptation to the aquatic environment

The mammalian transition from land to water required a series of adaptations to cope with the challenges of an aquatic lifestyle. Water is 800 times denser (Williams, 1999) and approximately 40 times more viscous than air (Reeb et al., 2007), which makes locomotion more challenging. Aquatic mammals experience greater pressure, which increases 1atm every 10m below the surface; therefore for deep divers, the pressure is constantly fluctuating (Reeb et al., 2007). Water has greater cooling power, with a thermal conductivity 25 times greater than that of air (Nienaber et al., 2010; Schmidt-Nielsen, 1975; Scholander et al., 1950b). This contrasting scenario, plus the fact that water is significantly colder than their core temperature of ~37°C (Scholander et al., 1950a), required that mammals needed increased structural and thermal insulation in order to adapt to the aquatic medium.

Aquatic mammals have developed a prominent layer of fat beneath the skin, called blubber, which serves as an insulator (Iverson, 2009a; Reeb et al., 2007). Blubber is an adaptation to the aquatic medium since it is different from the adipose tissue in terrestrial mammals. This tissue corresponds to the hypodermis of the skin (the deepest layer), which has thickened, and consists of loose connective tissue which is composed of fat cells (adipocytes), and is greatly enriched in collagen and elastic fibres (Ackman et al., 1975; Berta et al., 2006; Hamilton et al., 2004; Reeb et al., 2007). Blubber is a pliant biocomposite (Hamilton et al., 2004) containing collagen and elastic fibres which form the skeleton of the connective tissue (Ackman et al., 1975). This makes blubber a good outer body covering (Hamilton et al., 2004) as it allows relatively large-scale deformation for better streamlining, and protects the body from the extra pressure they experience in the aquatic medium.

Blubber is anatomically and biochemically adapted to serve as an efficient and adjustable thermal insulator. It contains an intertwined network of veins and arteries that allow larger and swifter blood supply, which is important to the thermoregulatory process (Ackman et al., 1965; Castellini, 2009; Iverson, 2009a).

The blubber layer is one of the most well-known and universal characteristics of aquatic mammals, but since species differ in the degree to which they are adapted to this habitat, these differences are also reflected in their blubber. Mammals returned to the water more than 60 million years ago (Williams, 1999). The first mammals to enter the water were cetaceans and sirenians in the later Early Eocene (Uhen, 2007). The blubber layer in cetaceans is extensive and is the only insulator. In blue whales, *Balaenoptera musculus*, for example, the blubber can reach a thickness of 50 cm in some areas of the body (Slijper and Harrison, 1979). Similarly, sirenians rely completely on their blubber as the only thermal insulator as their fur is sparse and has a tactile instead of insulating role (Reep et al., 2002). Pinnipeds were the next group to enter the water, in the late Oligocene (Uhen, 2007). They have a thick blubber layer which they use as an insulator in combination with fur (Liwanag et al., 2012b). Polar bears, *Ursus maritimus*, and otters are thought to be very recent entries into the aquatic environment compared to their counterparts (Uhen, 2007). In the polar bear, there is a thick layer of fat beneath the skin that appears to be blubber (Øritsland, 1970a), but does correspond to a specific anatomical adaptation as it is not different from the fat of other terrestrial carnivores (Iverson, 2009a). The fat layer of polar bears arises from thickening and lateral expansion of superficial depots, which are also present in slender terrestrial mammals but on a smaller scale (Pond et al., 1992). In the otter, although the fat layer is variable in size, particularly in gestating and lactating females (Chinn et al., 2016), it is minimal

compared to other aquatic mammals (Thometz et al., 2016); therefore they rely exclusively on their thick fur to keep warm (Davis et al., 1988; Ling, 1974).

Blubber is an efficient insulator due to its low thermal conductivity. The insulation provided by the blubber layer depends on its thickness, lipid and water content, its peripheral blood flow, and biochemical composition (Berta et al., 2006; Liwanag et al., 2012b). Blubber also plays an important role in a variety of other functions; it serves as the primary storage site for energy, it creates a streamlined body shape since it reduces drag and minimises energy expenditure while swimming, and it aids in buoyancy control (Castellini et al., 2009; McClelland et al., 2012; Pabst et al., 1999; Samuel and Worthy, 2004). Due to the many roles played by this tissue, the study of its biochemical compounds can provide insights into several life aspects of an aquatic mammal that are otherwise difficult to study (Iverson, 2009a).

1.2 The structural and functional units of the blubber

The many functions of blubber are reliant upon its lipid content (Hamilton et al., 2004). Irrespective of the nature of the food, lipids are generally stored in their blubber when food intake exceeds the energy used. Contrarily, when the amount of food is insufficient, these stored lipids are used to cover the remaining energy requirement (Schmidt-Nielsen, 1975). Lipids are not only a global energy currency but they also have a great variety of physiological roles (Guschina and Harwood, 2009), supplying essential compounds for general metabolic functioning, reproduction and somatic growth (Kainz and Fisk, 2009).

Lipids are stored in the fat cells making up the blubber, mostly in the form of triacylglycerols, which are storage lipids that serve as high-energy sources (Kainz and

Fisk, 2009). Triacylglycerols are compounds containing three fatty acids (FAs) attached to a glycerol molecule (Fig. 1.1; Raclot et al., 1998). Thus, FAs are the "building blocks" of lipids (Iverson, 2009b) and the main fuel for mammalian muscle metabolism (Schmidt-Nielsen, 1975).

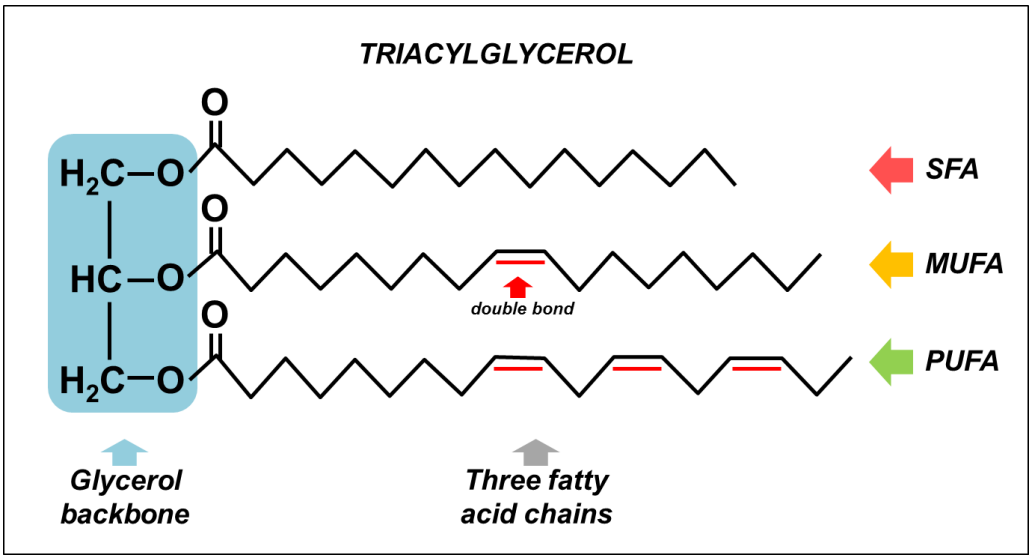


Figure 1.1. The triacylglycerol molecule consists of one glycerol backbone attached to three fatty acid (FA) molecules. In this diagram three FAs are represented, a saturated FA (SFA) without double bonds between carbon atoms; a monounsaturated FA (MUFA) with one double bond; and a polyunsaturated FA (PUFA) with two or more double bonds.

Fatty acids are compounds that contain even numbers of carbon atoms in straight chains (Fig. 1.1; Christie, 2003). In animal tissues, the common FAs vary in chain-length from 14 to 22, and in number of double bonds from one to six (Dalsgaard et al., 2003).

The number of double bonds determines the degree of desaturation of the molecule. Saturated FAs (SFAs) do not contain any double bond between carbon atoms; therefore they are "saturated" with hydrogen atoms (Fig. 1.1; Gunstone, 1996). Monounsaturated FAs (MUFAs) are those that have one carbon-carbon double bond, which may be

present in different positions (Christie, 2003). Polyunsaturated Fatty Acids (PUFAs) are compounds with two or more double bonds. PUFAs are mostly produced at low trophic levels by plants, bacteria, and phytoplankton and they are essential for all higher organisms (Christie, 2003; Sargent, 1976).

Here, FAs will be named using the following notation: A:BωX (i.e., 18:1ω9), where A indicates the number of carbon atoms in the molecule, B is the number of double bonds and X is the position of the first double bond relative to the terminal methyl group of the molecule (Fig. 1.2).

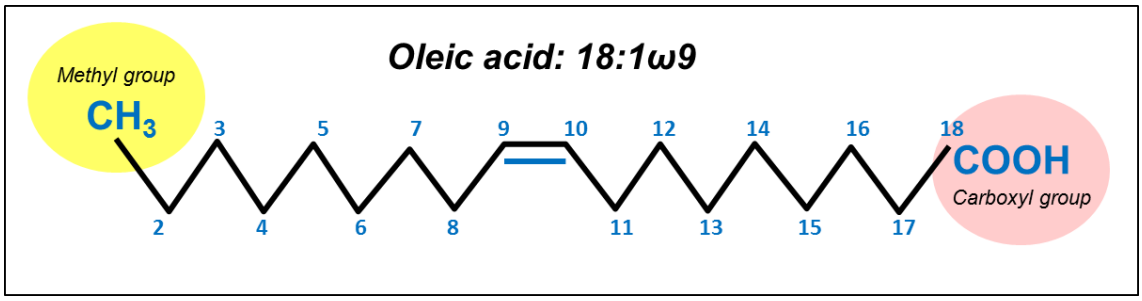


Figure 1.2. Diagram of oleic acid; a MUFA containing 18 carbon atoms. The position of carbon atoms is counted relative to the methyl end (carbon number one). Thus, in this example the position of the double bond is ω9.

1.3 Fatty acid analysis and dietary studies: the theory

The FA composition of an animal is markedly affected by dietary fats (Raclot, 2003). This has led to the use of FA analysis to infer the diet of those animals that are difficult to study *in situ*. Specifically, the analysis of the blubber FA composition has attracted considerable scientific interest for its potential use as trophic markers.

A trophic marker is a compound whose origin can be uniquely and easily tracked, that is not selectively processed during food ingestion, and that is metabolically stable. These

characteristics allow trophic markers to be transferred from one trophic level to the next in a predictable qualitative and quantitative manner (Dalsgaard et al., 2003). Although FAs do not comply with all these conditions, they are good candidates in the absence of many other alternatives.

Iverson (2009b) states that FAs can be used as tracers of diets and marine food-web structure due to these three reasons: 1) depending on the taxa or even species, organisms have biochemical limitations on FA synthesis or modification; 2) unlike other dietary compounds, FAs are generally taken up by tissues in their original form; and 3) FAs accumulate in the body (i.e.: adipose tissues) thus they can provide information on dietary intake over different periods of time depending on the organism, its energy intake and storage rates. Thus, the use of FAs as trophic markers in aquatic mammals is based on the principle that particular FAs present in the prey can be transferred conservatively into the blubber of the predator (Iverson, 1993).

In monogastric (one single-chambered stomach) mammals, during the digestion of triacylglycerol, FAs are separated from the glycerol backbone (Fig. 1.1) and are transferred conservatively through the bloodstream. These FAs pass through the mucosal wall of the small intestine, and reform into triacylglycerol (Budge et al., 2006; Iverson, 2009a). Then, triacylglycerols are transported by chylomicrons through the blood and taken up by the tissues the same way (Budge et al., 2006). Thus, FAs are transferred conservatively through the digestive process with no modification from the original FAs in the prey (Dalsgaard et al., 2003; Iverson, 2009a). Therefore, the identification of these FAs through FA analysis of the blubber can provide information on the type of prey consumed.

This occurs because the number of FAs that can be biosynthesized or modified by animals is quite limited (Dalsgaard et al., 2003). Mammals are usually restricted to

synthesise chains of 16 or 18 carbon atoms and, at most, one double bond (Iverson, 2009a). They do not have the ability to insert double bonds in positions closer than the 7th carbon from the methyl end (ω7) of the FA molecule (Fig. 1.3; Gladyshev et al., 2009). Most FAs are synthesized at the base of the food web by primary producers (Bromaghin et al., 2012; Budge et al., 2006; Colombo et al., 2016). For example, the *de novo* (made from simple molecules) synthesis of ω3 PUFAs in aquatic ecosystems is generally restricted to algae (Fig. 1.3; Kainz and Fisk, 2009) and therefore, some FAs such as linolenic acid (18:3ω3), can only be obtained from diet (Gladyshev et al., 2009). Thus, the presence of specific FAs found in marine mammal blubber have a dietary origin that can be traced (Iverson, 2009b). Not only specific FAs can be used for dietary inference; the array of FAs, or FA signature, can be substantially different among various species and hence can be used to distinguish between prey types and geographical location (Iverson, 1993).

Compared to the traditional gut content analyses which only reveals the last food intake; FA analysis can provide dietary information over a longer period of time (Dalsgaard et al., 2003; Tucker et al., 2009; Waugh et al., 2012b), which can be ecologically more significant. Several studies have demonstrated that FA analysis may reflect the diet patterns of aquatic mammals. Iverson et al. (1997) studied the FA composition of the milk in lactating fur seals, *Arctocephalus gazella*, from South Georgia. The FA patterns indicated that three different prey types were consumed by the mothers during the lactation period; this data coincided with scat content analysis. Furthermore, Tucker et al. (2009) analysed the FA profiles of harp seals, *Pagophilus groenlandicus*, over a period of ten years and the data coincided with large variations in prey availability. These are some examples that indicate that FAs have the potential to be an effective way to predict dietary intake.

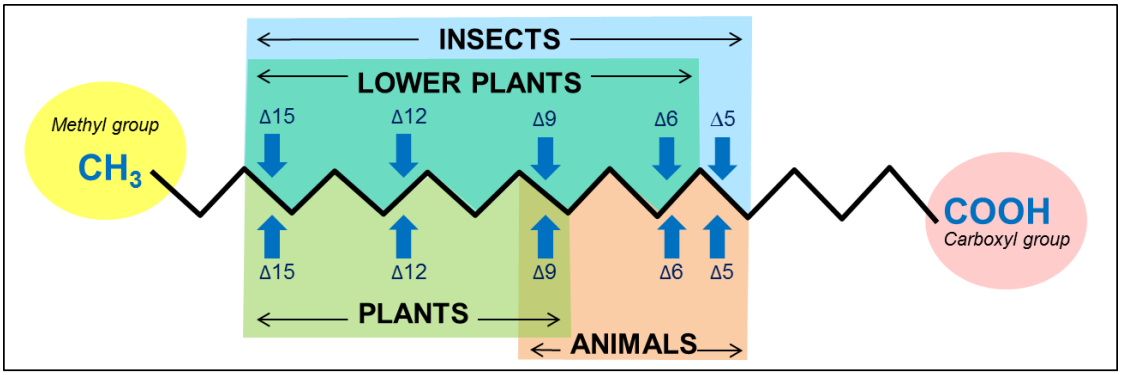


Figure 1.3. Position of carbon atoms where animals, plants, insects and lower plants (most marine algal species) have the ability to insert a desaturation (double bond). Here the delta (Δ) nomenclature is used, which describes a bond position relative to the carboxyl (number one) carbon of the chain. Diagram adapted from Cook and McMAster (2002).

1.4 Intrinsic physiological factors

Fatty acids can be either obtained from diet, or synthesized *de novo* through metabolic processes (Miyazaki and Ntambi, 2008). Due to the fact that only certain types of FAs can be made by mammals, it is possible to distinguish between dietary and non-dietary FAs (Iverson et al., 2004). Dietary FAs are those that could only be derived from the diet as animals are unable to synthesize them, such as most PUFAs (Iverson, 1993; Käkëla and Hyvärinen, 1996a). Non-dietary FAs are those that can be readily synthesized by the animal; most of them are SFAs and MUFAs, although some of them may be partially obtained from diet as well (Iverson, 1993; Iverson et al., 2004). Therefore, the concept of FAs traveling up the food chain without change is not as straightforward as it first appears.

Grahl-Nielsen *et al.* (2011) compared the FA composition of harp seals and their prey, but there was no strong resemblance between predator and prey FAs. In light of this, the authors suggest that the composition of blubber may not be defined only by the prey, but also by intrinsic metabolic factors.

Depending on the nutritional condition of the consumer, lipid deposition and mobilization rates may vary (Raclot, 2003). Different organisms have specific lipid requirements and/or different ability to gain physiological benefits from certain FAs (Kainz and Fisk, 2009). This implies that certain FAs obtained from the prey may be immediately utilised by the organism; hence their presence in the blubber of the predator may be underestimated. Additionally, an organism experiencing a dietary surplus of energy may modify at least part of their FAs to suit particular physiological needs (Dalsgaard et al., 2003; Strandberg et al., 2008). For example, Wheatley et al. (2007) showed that some FAs are selectively mobilised during the lactation period of Weddell seals, *Leptonychotes weddellii*. Other FAs not utilised by the organism may be deposited in the blubber and therefore accumulate in greater amounts. This, indeed, would lead to inaccurate dietary predictions as the FAs in the blubber will not exactly reflect those of the prey.

1.5 Stratification of blubber fatty acids in marine mammals

When collecting blubber samples for dietary studies, it is necessary to consider the potential existence of vertical variation of FAs throughout the blubber layer. The section just beneath the skin, hereafter referred to as the outer blubber layer, is usually cooler than the section closer to the underlying muscle, the inner blubber layer. This thermal gradient is accompanied by a biochemical gradient, where FAs from the outer layer differ in quantity from those of the inner layer. This difference, also named FA stratification, has been found in most aquatic mammals. Cetaceans (Koopman, 2007; Koopman et al., 1996; Krahn et al., 2004; Olsen and Grahl-Nielsen, 2003), sirenians (Skoglund et al., 2010; West et al., 1979b) and pinnipeds (Arnould et al., 2005; Best et al., 2003; Grahl-Nielsen et al., 2005; Wheatley et al., 2007) have been reported to have

a stratified blubber, although in otariids this stratification is less pronounced compared to other marine mammals (Arnould et al., 2005; Lambert et al., 2013).

The variation in the composition of blubber suggests that some components of the blubber are being synthesized independent of diet and that these two layers have different physiological functions (Wheatley et al., 2007). The inner blubber layer is thought to be more metabolically active since it is closer to the body core; therefore lipid metabolism may be easier and more efficient (Koopman, 2007). Struntz et al. (2004) compared the composition of blubber between robust and emaciated adults of bottlenose dolphins, *Tursiops truncatus*, and observed that the most marked reduction in size of fat cells occurred in the middle and inner blubber layers. Similarly, FA mobilisation during lactation of Weddell seals occurred mainly in the inner layer (Wheatley et al., 2007). In fin whales, *Balaenoptera physalus*, changes in the reproductive cycle produced alteration in lipid content in the inner layer, whereas the outer layer was insensitive to these changes (Aguilar and Borrell, 1990). These studies demonstrate that the inner layer is more metabolically active (i.e. experience periods of catabolism and anabolism); therefore dietary processes related to energy storage are likely to take place in this section of the blubber.

The outer layer has been found to be more stable than the inner layer. It is highly probable that metabolic reactions related to diet do not occur in this section of blubber (Koopman, 2007). This layer may serve a more structural role such as streamlining (Koopman et al., 2002; McClelland et al., 2012; Struntz et al., 2004). Additionally, it has been suggested that the outer layer is linked to other longer-term processes rather than feeding. For instance, Grahl-Nielsen et al. (2011) found a positive correlation between age and some FAs present in the outer blubber layer of harp seals. This

suggests that FA composition may be a result of a cumulative process throughout the lifetime of an animal.

The stratification of FAs can have implications for dietary studies. Depending on the section of the blubber core utilised, dietary predictions can differ in accuracy. Grahl-Nielsen (2009) states that the use of whole blubber core is not appropriate for dietary studies. When inner and outer layers are analysed separately, the use of the inner layer is recommended for dietary inference (Grahl-Nielsen et al., 2011).

1.6 The role of fatty acids in thermoregulation

The effect of temperature on FAs has been well documented at low trophic levels. The most commonly observed change in FAs following a temperature shift is an alteration in desaturation. The degree of desaturation of a FA is given by its number of double bonds within the chain (Miyazaki and Ntambi, 2008). As the number of double bonds increases, the solidifying point of the FA decreases (Fig. 1.4), thus tissues will remain fluid at cold temperatures. Algae, for instance, generally increase their relative amount of FA desaturation when exposed to lower environmental temperatures (Guschina and Harwood, 2009; Thompson, 1996). The copepod *Arcatia spp* was found to have higher MUFA in winter and spring, whereas during summer and autumn it had higher SFA (Jeffries, 1969). A similar effect of temperature was found in carp fish (Tiku et al., 1996). This alteration in desaturation could also impact the quality of FA transferred to consumers (Kainz and Fisk, 2009). In aquatic mammals; however, the effect of thermal adaptation in FAs has been studied to a lesser extent.

Because the blubber layer is in direct contact with the surrounding environment, the effect of environmental temperature would be most noticeable. This suggests that the

outer layer plays a role related to thermoregulation. In the case of cold-water inhabitants, the outer layer may reach very low temperatures (i.e.: 5°C in an arctic seal; Käkëla and Hyvärinen, 1996a), which could influence the type of FAs stored. It has been reported that some changes in the FAs making up the adipose tissue can be driven by thermal acclimatization (Harlow and Varnell, 1980; Käkëla and Hyvärinen, 1996a; Käkëla and Hyvärinen, 1996b; Koopman, 2007; Liwanag et al., 2012b). For instance, Käkëla and Hyvärinen (1996a) documented higher proportions of MUFAs in the cold extremities of northern aquatic and terrestrial mammals, compared to the rest of the body. Price et al. (2013) investigated the adipose tissue of 13-lined ground squirrels, *Ictidomys tridecemlineatus*, and found that these mammals exhibited a higher MUFA-to-SFA ratio in winter than during summer. Changes in the solidifying point of adipose tissues have been reported for domestic pigs when they were kept at different ambient temperatures (Henriques & Hansen, 1901, cited in:Irving et al., 1957). These findings suggest that some changes in blubber FAs in mammals may be driven by their thermoregulatory demands.

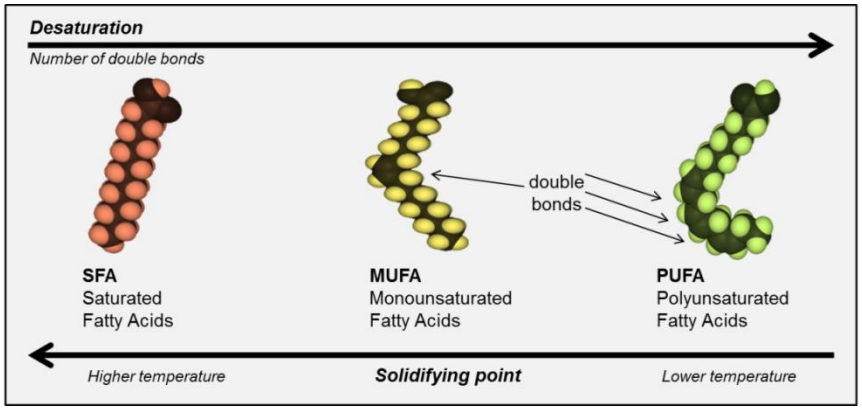


Figure 1.4. Desaturation of FAs. As the number of double bonds increases, molecules become more desaturated and the solidifying point (temperature at which they freeze) decreases. Thus, SFAs solidify at higher temperatures than MUFAs and PUFAs and, therefore, adipose tissues with higher proportions of SFAs (lower desaturation) would not be suitable for extremely cold environments.

1.7 Thesis outline

The composition and organization of lipids can have both subtle and obvious effects on an organism's health and functions. These effects can scale to higher levels, affecting populations, communities and ecosystems (Guschina and Harwood, 2009). The study of these lipids can; therefore, provide valuable information in aspects of the ecology of an organism or even of the whole ecosystem. Here, I investigate factors such as blubber FA stratification and FAs as dietary tracers in two phocid species, and the role of FAs in mammalian thermoregulation. The aim of this study was to understand how the FA composition of the blubber layer relates to its physiological roles as energy reservoir and thermal barrier.

Chapter 2 investigates the patterns of FA composition in the blubber of leopard seals, *Hydrurga leptonyx*, and its implications for dietary analyses. This is the first study, to the author's knowledge, that analyses FA composition and stratification in this Antarctic marine mammal. In order to identify whether the blubber layer of this top predator was stratified, I analyse inner, middle and outer layers separately. To investigate predator-prey relationships, I compare the FA composition of inner and outer layers to that of the potential prey species of leopard seals. With this analysis I have been able to identify whether the use of inner and outer layers would infer dietary items with similar accuracy. This chapter has been published in 2016 in the Journal of Experimental Biology and Ecology (Guerrero et al., 2016).

In Chapter 3, I investigate the FA stratification in another Antarctic predator, the crabeater seal, *Lobodon carcinophagus*. To my knowledge, this is the first description of the blubber FA composition of this mammal. This time, I analyse only outer and inner layers separately to identify the degree of FA stratification. I discuss the implications of the stratification of blubber for crabeater seals in comparison to other

marine mammals. Crabeater seals, as specialist consumers, are ideal for trophic studies as Antarctic krill comprises more than 90% of their diet (Hückstädt et al., 2012); therefore, the blubber of crabeater seals potentially reflects the diet more closely than in other, more generalist species. Here, I analyse the diet of these seals using both the whole array of FAs and only dietary FAs to determine which analysis will provide a better outcome. I also conduct a dietary analysis using either outer or inner layer FAs to determine how they perform relative to each other in predicting diet.

In order to understand how the environmental temperature can influence blubber FAs, in Chapter 4 I conduct a meta-analysis where, based mostly on literature data, I compare the degree of FA desaturation of 48 mammalian species, which are terrestrial, semi-aquatic or fully-aquatic. I also test how the presence of fur, another insulator, could impact the role of blubber FAs. This study provides interesting insights into the role of blubber FAs in the thermoregulatory strategy of mammals facing different environmental conditions.

In Chapter 5 I analyse several parameters of the insulating system of three Antarctic seals: the leopard, the Weddell and the crabeater seal. These three species inhabit the same environment but possess different morphological characteristics, which may influence their thermoregulatory strategy. I study the relationship between blubber thickness, blubber FA desaturation, fur density and some morphometric parameters such as body size, slimness, and limb ratio. This study shows that even living in the same habitat, different species do not necessarily cope with the extreme cold in the same manner.

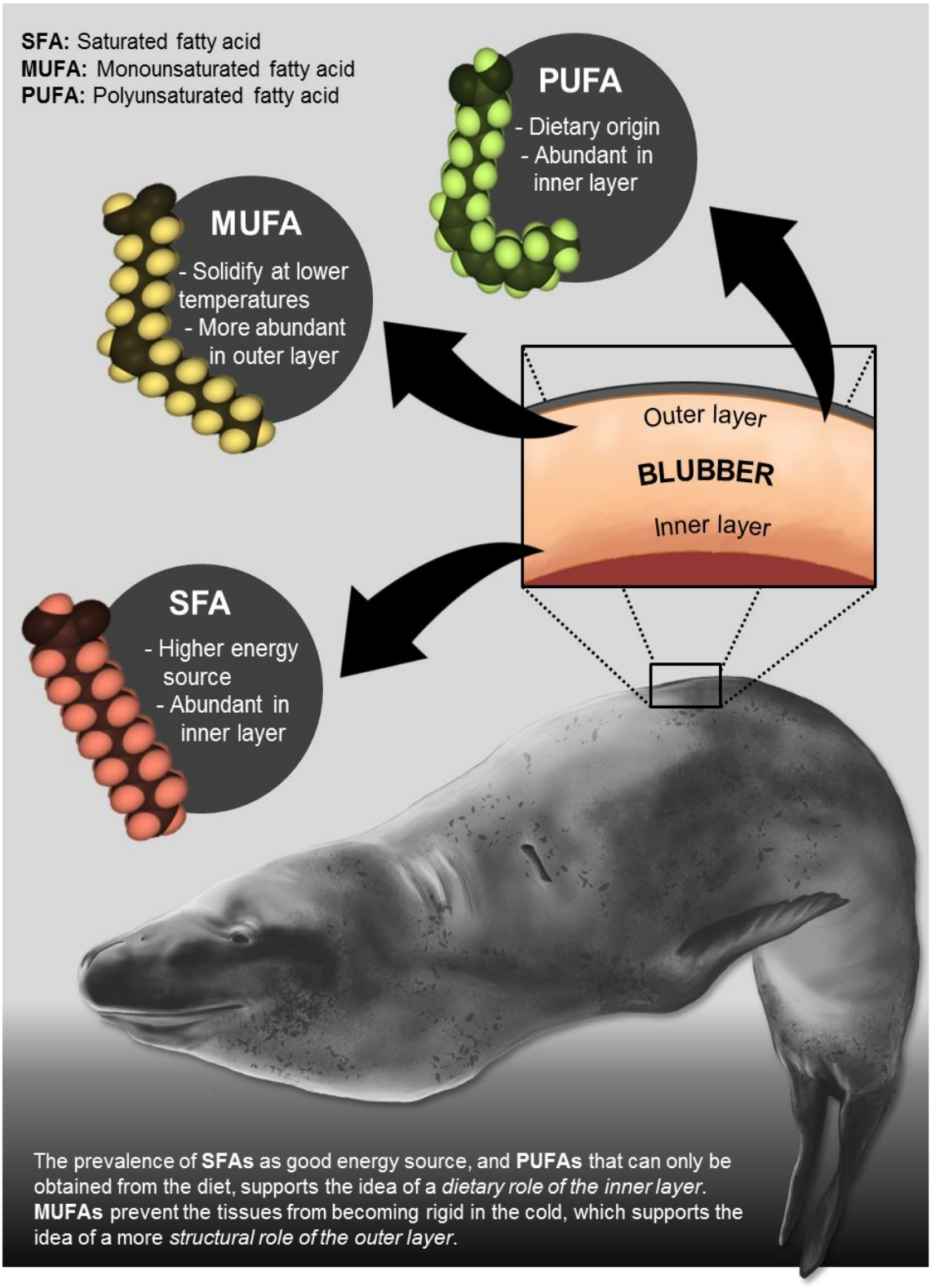
CHAPTER 2

Vertical fatty acid composition in the blubber of leopard seals and the implications for dietary analysis

This chapter has been previously published (see Appendix 1) in the *Journal of Experimental Marine Biology and Ecology,* as*:*

Guerrero, A.I., Negrete, J., Márquez, M.E.I., Mennucci, J., Zaman, K., Rogers, T.L., 2016. Vertical fatty acid composition in the blubber of leopard seals and the implications for dietary analysis. J. Exp. Mar. Biol. Ecol. 478, 54-61.

2.1 Graphical Abstract



2.2 Abstract

The analysis of blubber FAs is a useful tool to infer diet of mammals that live in remote regions where year-round studies are difficult. The FAs may not be distributed uniformly within the blubber, which can have implications for dietary predictive studies. The aim of this study was to determine the FA composition in the blubber core of the Antarctic leopard seal and evaluate the potential implications of FA stratification for dietary inference. The blubber cores of 24 seals were sub-sectioned into outer, middle and inner layers and their FAs were compared to those of their potential prey species. A vertical variation in FA composition was found across the whole blubber core of the leopard seal. Seventeen FAs were found at greater than trace amounts (>0.5%) being the most abundant: 18:1ω9, 16:1, 22:6ω3, 16:0 and 18:1ω7, which accounted for approximately 70% of the total FA. Almost all FAs had a continuous gradient through the blubber. Principal Component Analysis confirmed separation between inner and outer layers while the middle layer was a transition. The stratification of the leopard seal blubber was similar to the general pattern observed in a variety of marine species: MUFAs dominated the three layers being more abundant in the outer layer whereas PUFAs and SFAs were more abundant in the inner layer. PUFAs are of dietary origin and SFAs are chemically inert so they can be used as a long-term reserve, which suggest that the inner layer is the site of deposition of the FAs obtained from diet. The influence of prey on the composition of the leopard seals' blubber was clearer in the inner layer, although neither outer nor inner layers exactly matched the FAs of the potential prey. This suggests that there are other factors influencing the FA composition of this predator.

2.3 Introduction

Traditional methods used in foraging ecology, such as scat and stomach analyses, although are useful to determine overall trends (Hoberecht, 2006), they have known biases and limitations associated with incomplete consumption of prey items, gut passage rate and differential degradation of prey remains (Dellinger and Trillmich, 1988; Gales and Cheal, 1992). Blubber FA analysis, on the other hand, can provide a long-term indication of diet history (Bradshaw et al., 2003; Dahl et al., 2000); therefore it can be used to obtain more complete data on diet composition than traditional methods.

The FA composition of blubber, however, is not exactly identical to that of the diet (Cooper et al., 2005; Grahl-Nielsen, 2009) since it may be regulated by other factors (Grahl-Nielsen and Mjaavatten, 1991). Additionally, the FA stratification of blubber complicates the investigation of foraging ecology through this method. Therefore, before using the blubber for obtaining dietary information, it is important to determine the species-specific FA composition through the blubber core.

Different studies have taken contrasting approaches to infer diet; some use either the outer component only (Herman et al., 2005; Waugh et al., 2012a), or a section across the whole blubber (Bradshaw et al., 2003; Meynier et al., 2008; Newland et al., 2009), while other studies recommend the use of the inner component (Grahl-Nielsen et al., 2005; Olsen and Grahl-Nielsen, 2003; Skoglund et al., 2010). The leopard seal is an ideal model to study foraging ecology using FA analysis, as they live in the remote Antarctic pack ice where long-term dietary studies are very difficult to carry out. They have a dispersed distribution (Forcada et al., 2012; Rogers et al., 2013; Southwell et al., 2008), individuals travel widely (Meade et al., 2015; Rogers et al., 2005) and haul out on the drifting ice floes (Rogers and Bryden, 1997; Rogers et al., 2013; Southwell et al.,

2003) making year-round studies difficult. Their feeding ecology has been determined by different means, including direct hunting observations (Ainley et al., 2005; Hiruki et al., 1999; Penney and Lowry, 1967; Rogers and Bryden, 1995), stomach contents (Siniff and Stone, 1985), scats (Casaux et al., 2009; Green and Williams, 1986; Hall-Aspland and Rogers, 2007; Hall-Aspland and Rogers, 2004; Rogers and Bryden, 1995; Walker et al., 1998) and stable isotope analyses (Hall-Aspland et al., 2005).

Leopard seals fast during the pupping season, which takes place in November (Siniff, 1991). By summer, when samples for this study were collected, seals are feeding again (Hall-Aspland and Rogers, 2004; Siniff, 1991) and hence storing surplus energy in their blubbers, which is an ideal time to assess dietary influence. This study will identify initially whether the blubber of the leopard seal is stratified by establishing the vertical variation in FA composition across whole blubber cores. The influence of FA stratification on dietary studies will be tested by comparing how FA composition of the inner and outer blubber layers perform in inferring individual diet and trophic levels.

2.4 Materials and methods

2.4.1 Sample collection

In total, 24 leopard seals, 9 females and 15 males, were sampled off the Danco Coast, Western Antarctic Peninsula (64°09′ S 60°57′ W) during the austral summer (February) of 2008 and 2009. Seals hauled out on sea ice were immobilised using a Tele-inject air gun darting system using tiletamine/zolazepam (Higgins et al., 2002). Following immobilisation, sex, standard length (SL, straight line nose to tail) and pectoral girth (PG) were recorded and 8 mm diameter biopsies containing whole cores of blubber (from the skin to the muscle layer) were collected from the mid-dorsal surface. All

individuals were adults and the body length averaged 280.8 ± 18.3 cm in females and 269.3 ± 19.1 cm in males. In the laboratory, the blubber cores were separated into three sections (inner, middle and outer layers), as shown in Figure 2.1.

Each sample was stored in airtight vials and frozen at -20°C for up to 60 days, while in the field, and then at -80°C for further analyses. The immobilisation and sampling of leopard seals in the Antarctic Specially Protected Area No. 134 was approved by the Dirección Nacional del Antártico, Buenos Aires, Argentina, and performed according to the SCAR Code of Conduct for Animal Experiments under UNSW Animal Care and Ethics Committee (Protocols 08/103B and 11/112A).

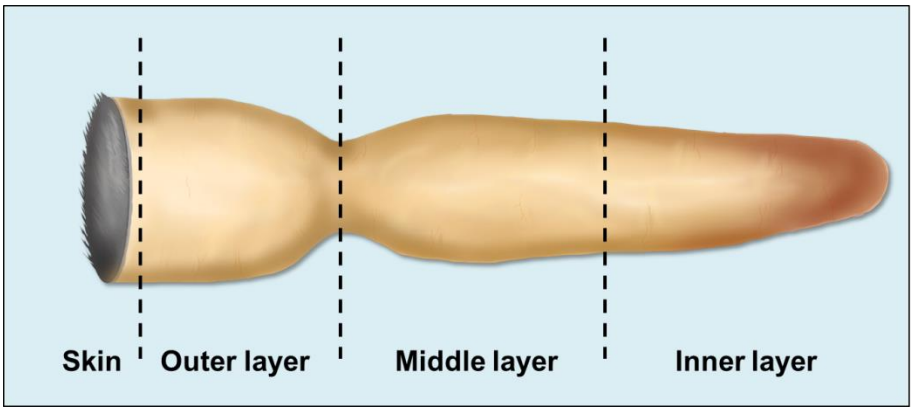


Figure 2.1. Sectioning of the blubber core. The outer layer was taken between the skin and the narrowest part of the blubber column. The remaining sample was divided into two sections: the middle layer (the closest to the outer layer) and the inner layer (the closest to the muscle).

2.4.2 Fatty acid analysis

Total lipid was extracted following a modified Folch et al. (1957) method. Approximately 0.3g – 0.5g of blubber was weighed and placed in 9ml of 2:1 chloroform: methanol with 50mg/L of butylated hydroxytoluene in test tubes with Teflon caps. Samples were macerated manually with a glass homogenizer until thin and

transparent, then vortexed for 20s, and allowed to soak overnight at 4ºC. To remove protein precipitates the homogenate was filtered into Teflon screw cap glass tubes, 2ml of water were added and the whole mixture was agitated and then allowed to separate into two phases. The lower phase containing lipids was carefully collected by siphoning/ pasture pipette and placed in Teflon lined screw cap tubes. The solvent (chloroform) was evaporated from lipid under nitrogen stream to avoid lipid oxidation.

Fatty acid methyl esters (FAMEs) were prepared directly from the extracted lipid, which was dissolved in 1.5 ml of boron trifluoride (10% in methanol) and 1.5 ml of toluene. The solution was capped under nitrogen and heated at 50°C overnight. Esters were extracted into hexane and stored in vial tubes for gas chromatography analysis.

Gas chromatography analyses were performed with Agilent 6850 Series GC System (Agilent Technologies, Santa Clara, CA, USA) equipped with a flame ionization detector. FAMEs were analysed using a 100m long fused carbon-silica capillary column (SP 2560 Column). The flow rate of the hydrogen carrier gas was set at an initial flow rate of 2.7 mL/min, at a pressure of 200 kPa. The injector and detector port temperatures were set at 260ºC. The column oven was initially held at 140°C for 5 min, then increased 4°C/min and then maintained at a temperature of 235°C. The total run time per cycle was 35 minutes. Peak areas and retention times were calculated (ChemStation Software, Rev. B.03.01; Agilent Technologies), and FAMEs were identified by comparison of retention times with a range of standards. The concentrations of individual FAs in each sample were converted to percentage contributions of the total FA.

2.4.3. Data analyses

To reduce analytical noise, FAs present in trace amounts (<0.5%) were excluded from statistical analyses. Thus, the number of FAs was reduced to 17. The largest FA proportion was more than 2000 times greater than the smallest FA proportion, so all values (x) were arcsine-transformed ($x' = \arcsin\sqrt{x}$) in order to reduce the heterogeneity of variance among groups. Normality and homogeneity of variance were checked with normality plots and with plots of residuals versus fitted values, respectively.

Due to the multivariate nature of FA profile data, Principal Component Analysis (PCA) was used to investigate patterns in FA association among the different individuals. In this manner the 17 variables could be described in two dimensions. Multivariate analysis of variance (MANOVA) was then carried out on the first 3 PC scores with sex, year, and blubber layers as factors.

In order to determine the degree of stratification in the blubber, a stratification index was calculated using the formula by Olsen and Grahl-Nielsen (2003):

$$SI = \frac{(F_o - F_i)}{\left(\frac{F_o + F_i}{2}\right)}$$

where F_o is the percentage concentration of each FA in the outer layer and F_i is the concentration in the inner layer. Thus, positive values of SI represent a greater amount of the FA in the outer layer and negative values indicate a greater presence in the inner layer.

A correlation between body condition and inner layer FAs was tested. Body condition was assessed using fineness ratio (Castellini and Kooyman, 1990) which was derived from standard body length and girth measurements.

Fineness ratio (*FR*) is an index to measure streamlining and hydrodynamic performance (Van den Hoff et al., 2005). Assuming that, when supported by water the seals body would have a roughly circular cross-section it is calculated as the standard body length (S*L*) divided by its maximum diameter (D_{max}):

$$FR = \frac{SL}{D_{max}}$$

All statistical tests have α level of statistical significance of 0.05 (SPSS Release 17.0, SPSS 2008). Where not identified, variability around the mean is standard deviation (SD).

2.4.4 Potential prey fatty acids

In order to test whether the use of inner or outer layers would lead to the same predictions of diet, a PCA for leopard seal blubber layers and potential prey FA composition was plotted. Additionally, their trophic level was investigated by using FA ratios of vaccenic acid/oleic acid (18:1ω7/18:1ω9) and eicosapentaenoic acid (EPA) / docosahexaenoic acid (DHA) (20:5ω3/22:6ω3).

Data of prey species was collated from published literature, which included species of fish (Connan et al., 2010; Lea et al., 2002; Mayzaud et al., 2011; Raclot et al., 1998), krill (Alonzo et al., 2005; Cripps et al., 1999; Phleger et al., 2002; Polito et al., 2012) and penguins (Speake et al., 1999; Tierney et al., 2008), collected in different geographical areas within the Southern Ocean between 1992 and 2009.

2.5 Results

2.5.1 Fatty acid stratification

Although 46 FAs were originally identified, only 17 (comprising 89.34% of total) were found consistently in all blubber layer samples and in proportions greater than 0.5%. The most abundant FAs, accounting for approximately 70% of total FA, were: 18:1ω9 (19-27%), 16:1 (10-17%), 22:6ω3 (6-9%), 16:0 (6-10%), and 18:1ω7 (7-8%) (Table 2.1; Fig. 2.2).

The FA profiles of the three layers were dominated by MUFAs (40-55%), followed by PUFAs (20-30%) and with smaller proportions of SFAs (12-19%) (Table 2.1, Fig. 2.3). Monounsaturated FAs were present at higher proportions in the outer blubber layer (55.7 ± 4.2%) decreasing to 40.1 ± 5.3% in the inner layer. Conversely, SFAs and PUFAs were present at higher proportions in the inner layer (19.3 ± 2.7% and 30.4 ± 3.8%, respectively) compared to the outer layer (11.9 ± 1.6% and 20.1 ± 5.0%, respectively).

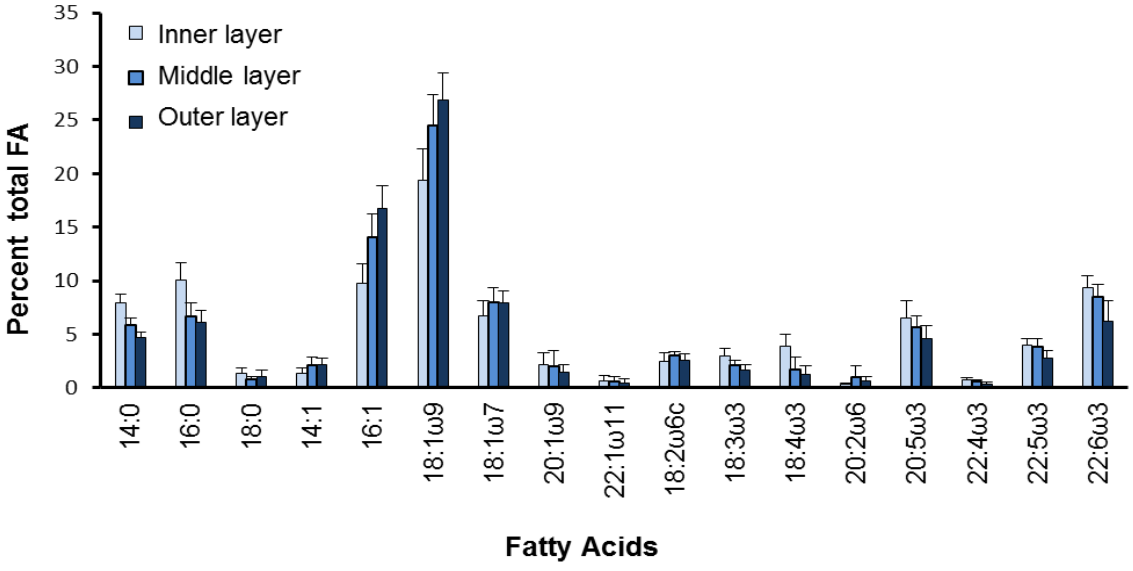


Figure 2.2. Proportion of individual FAs across blubber layers. Relative amounts (percent total) of 17 FAs in the inner, middle and outer blubber layers of leopard seals (n = 24).

Table 2.1 Fatty acid composition* (per cent total FA) of inner, middle and outer blubber layers in leopard seals (n=24).

	2008						2009					
	Female			Male			Female			Male		
	IL	**ML**	**OL**	**IL**	**ML**	**OL**	**IL**	**ML**	**OL**	**IL**	**ML**	**OL**
C14:0	8.21	6.12	4.99	7.41	5.67	4.77	7.97	5.98	4.49	8.25	5.75	4.61
C16:0	10.42	6.78	6.88	9.38	6.24	4.93	10.40	7.07	6.46	10.33	6.60	6.86
C18:0	1.22	0.89	1.41	1.12	0.88	0.62	1.58	0.67	0.96	1.38	0.73	1.49
Σ SFA	19.85	10.34	13.28	17.91	7.99	10.32	19.95	10.98	11.91	19.96	11.21	12.96
C14:1	1.50	2.04	2.17	1.42	2.39	2.63	1.31	1.76	1.73	1.34	2.28	1.97
C16:1	10.00	13.50	15.91	10.62	14.36	17.90	9.58	14.55	16.77	8.70	13.91	15.99
C18:1 ω 9	20.44	23.35	27.10	19.96	24.67	27.17	18.55	22.83	25.66	18.84	25.90	27.36
C18:1 ω 7	6.73	8.69	8.20	7.05	7.92	7.72	6.29	8.58	7.86	6.64	7.42	8.13
C20:1ω9	1.60	1.16	1.39	2.77	2.91	1.79	1.59	2.00	1.32	2.04	1.79	1.17
C22:1 ω 11	0.47	0.66	0.32	0.68	0.75	0.61	0.69	0.30	0.26	0.82	0.57	0.35
Σ MUFA	40.74	37.06	55.08	42.50	33.13	57.83	38.01	40.01	53.60	38.37	44.47	54.96
C18:2 ω 6	2.04	3.05	2.69	2.21	2.92	2.30	2.94	2.80	2.86	2.68	3.11	2.66
C18:3 ω 3	3.18	2.22	1.66	2.89	2.22	2.01	2.71	1.99	1.55	3.03	2.09	1.48
C18:4 ω 3	3.38	2.10	1.26	3.82	1.84	1.79	4.30	1.08	0.84	4.06	1.75	0.77
C20:2 ω 6	0.27	0.67	0.34	0.37	0.73	0.66	0.44	1.74	0.83	0.39	1.00	0.66
C20:5 ω 3	5.93	5.52	4.00	6.97	5.35	5.05	7.13	5.99	5.09	6.04	5.72	4.14
C22:4 ω 3	0.55	0.75	0.31	0.77	0.65	0.53	0.86	0.53	0.38	0.76	0.62	0.25
C22:5 ω 3	3.99	4.00	2.42	3.75	3.62	3.02	4.34	4.33	2.97	3.97	3.63	2.40
C22:6 ω 3	9.30	8.94	5.62	8.74	8.11	6.90	9.99	9.02	6.75	9.69	8.29	5.50
Σ PUFA	28.66	20.43	18.30	29.52	15.90	22.27	32.71	21.99	21.29	30.61	22.47	17.87

*Only those FA contributing >0.5% of the total are shown.

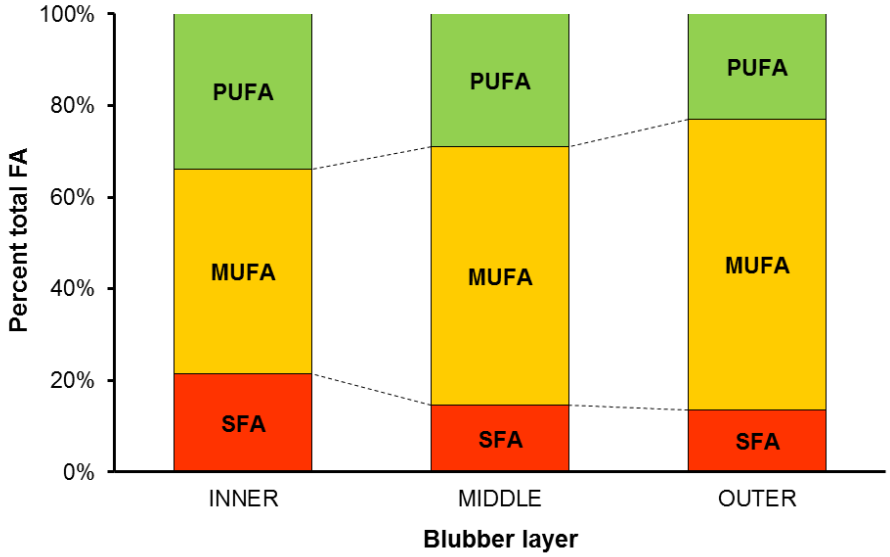


Figure 2.3. Fatty acid types across blubber layers. Mean proportion PUFAs, MUFAs, and SFAs from the inner, middle and outer blubber layers for leopard seals (n=24).

The first three components derived from the PCA accounted for 67% of the variation (PC1 43%, PC2 13%, PC3 11%) in FA composition among the samples. The bivariate plot (Fig. 2.4) shows inner and outer layers as separated groups, while the middle layer sits between them. The principal FA driving the segregation of the PC1 were the positive Eigenvalues for 18:4ω3, 18:3ω3, 14:0 and 22:6ω3 (greater abundance in the inner layer) and the negative Eigenvalues for 16:1, 18:1ω9 and 18:1ω7 (greater abundance in the outer layer). A MANOVA was carried out on the 3 PC scores and it confirmed the significant differences between blubber layers (*Wilks' λ* = 0.154, $P < 0.001$). Figure 2.4 also shows a group of seals, whose inner layer was separated from the others and was rather sitting with the middle layer group. This segregation of leopard seals inner blubber was not caused by differences in body condition of the seals, since there was no correlation between the fineness ratio ($t_{18} = 0.42$, $P = 0.67$) and the first PC for inner layer FAs across the 24 seals.

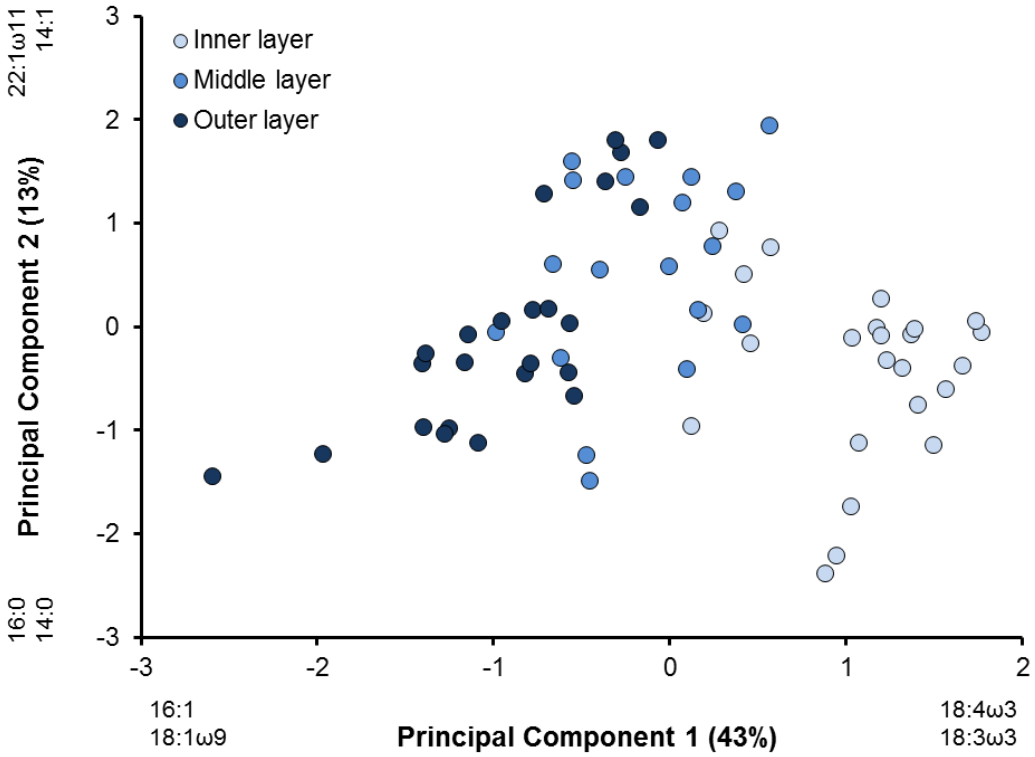


Figure 2.4. Vertical variation of FAs across the blubber layer of leopard seals. Principal component plot for the inner, middle and outer blubber layers of leopard seals (n=24).

The inner-outer difference per individual FA is represented by the stratification index (Fig. 2.5). The mean overall FA stratification index was 0.44 ± 0.22 and the maximum reached an absolute value of 1.11 for stearidonic acid (18:4ω3). While SFAs were more abundant in the inner layer, MUFAs were enriched in the outer layer. Most PUFAs showed an inner-layer trend, except for 18:2ω6c and 20:2ω6, which were in greater amounts in the outer layer.

There were no significant inter-sexual (*Wilks' λ* = 0.961, *P* = 0.482) or inter-yearly (Wilks' λ = 0.955, *P*=0.413) differences.

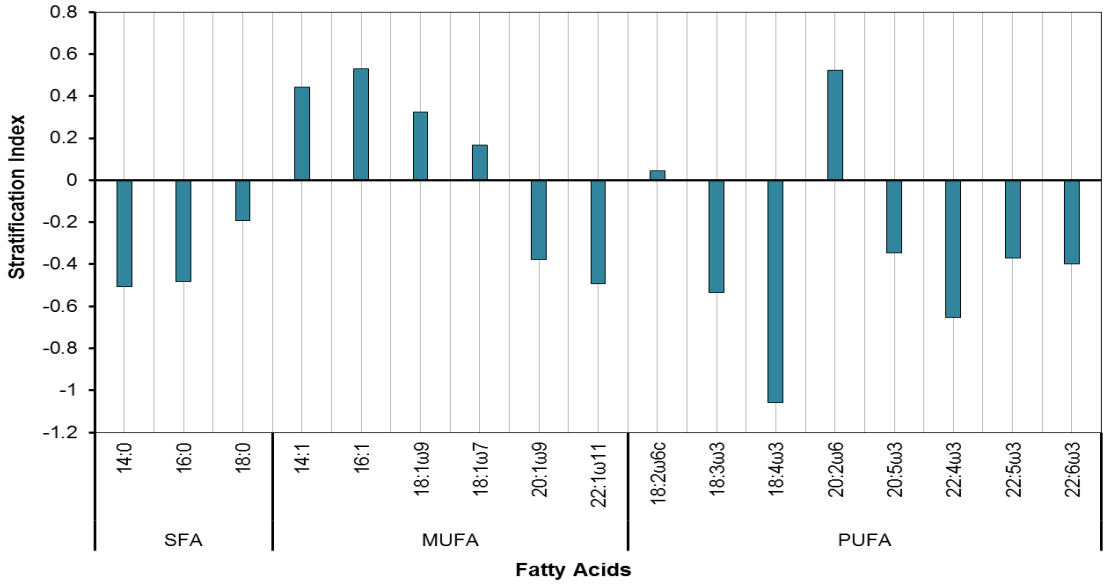


Figure 2.5. Stratification index for FAs in the leopard seal's blubber (n = 24). Positive values represent a greater amount of the FA in the outer layer and negative values indicate a greater presence in the inner layer.

2.5.2 Fatty acids and implications for dietary analysis

Principal component analysis comparing leopard seals to their potential prey species (Fig. 2.6) showed that the PC1 segregates prey species and predator, which segregation was influenced by higher amounts of 16:0 and 20:5ω3 in prey species and higher amounts of 18:1ω9 and 16:1 in leopard seals. The inner layer aligned more closely with prey species, compared to the outer layer. The second PC revealed similarity between fish and penguins, with higher amounts of 18:0 and 20:1ω9, and between leopard seals and krill species, due to the influence of positive Eigenvalues for 18:4ω3 and 14:0.

Figure 2.7 reveals that leopard seals, penguins and most fish species had low vaccenic acid/oleic acid ratios whereas most krill species had higher ratios. For leopard seals, penguins and most fish species, EPA/DHA ratios were low; but krill species showed a wide range of values.

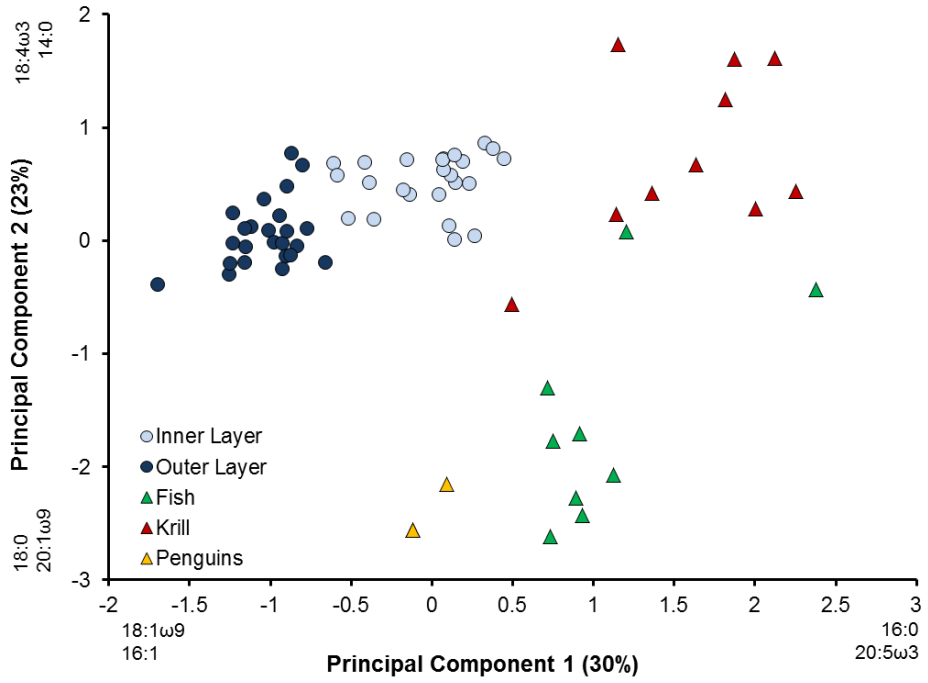


Figure 2.6. Principal Component plot of leopard seals inner and outer blubber layers (n = 24) and their potential prey species: fish, krill and penguins.

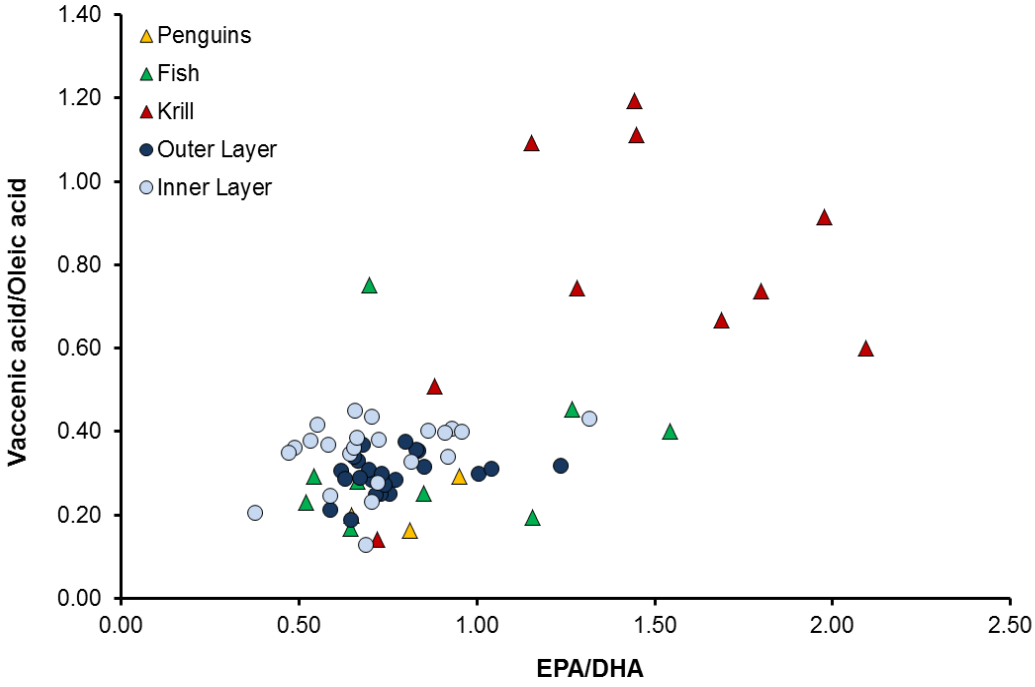


Figure 2.7. Vaccenic acid/oleic acid and eicosapentaenoic acid (EPA)/docosahexaenoic acid (DHA) ratios of leopard seals' inner and outer blubber layers, krill, fish and penguins.

2.6 Discussion

2.6.1 Stratification of fatty acids

Significant variations were observed in the FA composition between the outer and inner blubber layers, however rather than being discrete layers the FA composition changed as a gradient from the outer, middle to the inner layer through the blubber core. Blubber stratification has been reported for several phocid species including: harbour seals, *Phoca vitulina* (Andersen et al., 2004); southern elephant seals, *Mirounga leonina* (Best et al., 2003); Baikal seals, *Phoca sibirica*; ringed seals, *Pusa hispida* (Grahl-Nielsen et al., 2005; Strandberg et al., 2008); Weddell seals (Wheatley et al., 2007); and harp seals (Grahl-Nielsen et al., 2011). Winter and Nunn (1950) reported FA composition of the whole blubber core from three sites (the belly, back and neck) of the body of a single leopard seal. Similar FA compositions were reported between these three sites, but only four individual FAs were identified and stratification of the blubber core was not considered.

The stratification of blubber is attributed to the different roles that the layers provide, either related with thermoregulation for the outer layer and dietary processes for the inner layer (Arnould et al., 2005; Best et al., 2003; Koopman et al., 1996).

Saturated FAs were more abundant in the leopard seals' inner blubber layers than in the outer layers. In otariids there is little stratification of SFAs across the blubber core (Arnould et al., 2005; Lambert et al., 2013) but SFA stratification is pronounced in other phocids (Grahl-Nielsen et al., 2005; Strandberg et al., 2008), in the walrus, *Odobenus rosmarus*, (Skoglund et al., 2010) and in cetaceans (Budge et al., 2008; Olsen and Grahl-Nielsen, 2003). SFAs are thought to be important for long-term energy storage (Arriola et al., 2013) since they are comparatively chemically inert (Christie, 2003). In addition, having the highest melting point compared to the other groups of

FAs (Christie, 2003), the decreasing amount of SFAs towards the outer layer is reasonable as greater amounts of SFAs in the outer layer would make it very rigid, particularly at low temperatures, which would be unfavourable for movement and streamlining.

Leopard seals have higher quantities of MUFAs across all layers, but there are significantly more MUFAs in the outer blubber layer. This is similar to the MUFA distribution patterns across the blubber of other phocid seals, the harp (Grahl-Nielsen et al., 2011), ringed (Strandberg et al., 2008) and southern elephant (Best et al., 2003) seals as well as the otariid: the New Zealand sea lion, *Phocarctos hookeri* (Lambert et al., 2013). It has been suggested that because MUFAs have a lower melting point, relative to their saturated counterparts (Christie, 2003), their presence in the outer layer is important to maintain softness and fluidity under the skin and to help reduce heat loss from the body (Best et al., 2003). In the case of species that inhabited cold-water habitats, the temperature of the skin could be extremely low (e.g. 1°C in seals in icy water, Irving and Hart, 1957), which may limit enzymatic reactions (Koopman, 2007) impeding dietary processes in the outer layer.

Polyunsaturated FAs are more abundant in the leopard seals' inner blubber layer, which is not common across other seals, but has been reported for several cetacean species. They are in higher abundance in the inner blubber layer of dusky dolphins, *Lagenorhynchus obscurus* (Grahl-Nielsen et al., 2010), short-beaked common dolphins, *Delphinus delphis* (Smith and Worthy, 2006), harbour porpoises, *Phocoena phocoena* (Koopman et al., 1996), minke, *Balaenoptera acutorostrata* (Olsen and Grahl-Nielsen, 2003), and fin whales (Ruchonnet et al., 2006). Polyunsaturated FAs are similar in quantities across the inner and outer layer of the phocids: Weddell seals (Wheatley et al., 2007), ringed seals (Grahl-Nielsen et al., 2005; Strandberg et al., 2008); and the

otariids: the cape fur seal, *Arctocephalus pusillus* (Arnould et al., 2005) and New Zealand sea lion (Lambert et al., 2013); the Atlantic walrus (Skoglund et al., 2010); and the cetacean: the bowhead whale, *Balaena mysticetus* (Budge et al., 2008).

Higher presence of PUFAs in the inner blubber layer of the leopard seal may be due to their prevalence in the marine environment and the fact that they are usually of dietary origin (Hoberecht, 2006; Samuel and Worthy, 2004). Most PUFAs are essential FAs, which can only be acquired through diet (Arriola et al., 2013; Liwanag et al., 2012b). They are produced by primary producers and are tightly conserved with little catabolism in higher trophic levels (Dahl et al., 2000; Hoberecht, 2006). As PUFAs are susceptible to oxidative deterioration or autoxidation (Christie, 2003), they are rapidly available from the inner layer as a fuel source (Arriola et al., 2013). Despite this, the differences in PUFA distribution between marine mammals can be explained by distinct deposition and mobilisation of FAs through the depth of the blubber, which will also depend on their nutritional state.

Overall, the FA stratification index of leopard seals shows similar values to those found on other phocids (Grahl-Nielsen et al., 2005; Grahl-Nielsen et al., 2011; Strandberg et al., 2008; Strandberg et al., 2011) but are larger than those reported for otariids (Arnould et al., 2005; Lambert et al., 2013). This means that phocids have blubber with higher vertical variation than otariids. Pinnipeds in general, use a combination of blubber and fur as a mechanism of insulation (Liwanag et al., 2012b), but phocids use blubber as primary thermal insulator, since their fur has poor insulating properties when wet (Kvadsheim and Aarseth, 2002). Therefore, the higher level of blubber stratification in phocids could be a consequence of its better specialisation as an insulator: the outer layer may have differentiated to play an exclusive thermoregulatory role while the inner layer is the energy reserve ready to be utilised when the animal needs it.

2.6.2 Fatty acids and implications for dietary analyses

The two most abundant FAs, 18:1ω9 and 16:1, present in leopard seal's blubber are commonly abundant in marine mammals. They have been recorded in the highest proportions for phocids (Andersen et al., 2004; Grahl-Nielsen et al., 2005; Wheatley et al., 2007), otariids (Beck et al., 2007; Iverson et al., 1997; Lambert et al., 2013; Rosen and Tollit, 2012) and cetaceans (Budge et al., 2008; Quérouil et al., 2013; Waugh et al., 2012a). They are considered endogenous FAs (Herman et al., 2005) that do not have a dietary origin (Raclot et al., 1998), although it has also been suggested that high levels of these FA may be consistent with a carnivorous diet (Phleger et al., 2002).

Docosahexaenoic acid (DHA), 22:6ω3 was the third most abundant FA present in leopard seal's blubber, which is similar to other phocids (Arriola et al., 2013; Grahl-Nielsen et al., 2005; West et al., 1979a), otariids (Arnould et al., 2005; Beck et al., 2007; Iverson et al., 1997), Atlantic walrus (Skoglund et al., 2010), and some cetaceans (Budge et al., 2008; Quérouil et al., 2013; Ruchonnet et al., 2006; Smith and Worthy, 2006). This is an exogenous dietary FA (Herman et al., 2005) and hence a likely biomarker for the assessment of long-term dietary intakes (Raclot et al., 1998). Moreover, the higher presence of DHA in the inner stratus supports the thesis of a dietary role of this layer. This FA is typically very abundant in krill species (Tierney et al., 2008); therefore, the higher presence of DHA in leopard seals compared to other Antarctic seals such as southern elephant (Best et al., 2003) and Weddell seals (Wheatley et al., 2007) suggests that leopard seals are incorporating higher levels of krill in their diet. The other FA found in high amounts in krill, compared to the other prey species, is docosapentaenoic acid, 22:5ω3 (Tierney et al., 2008), which is also more abundant in the leopard seals than the previously mentioned Antarctic seals (Best et al., 2003; Wheatley et al., 2007).

Gondoic acid, 20:1ω9, was present in small (~2%) quantities in the inner blubber of the leopard seal compared to other phocid seals; such as the southern elephant (Best et al., 2003), harbour (Andersen et al., 2004) and ringed (Grahl-Nielsen et al., 2005; Strandberg et al., 2008) seals; where it corresponds to 10 to 15% of the total FA in the blubber. This FA has a dietary origin and is characteristic of many teleost species but it is found in very low levels in krill (Iverson et al., 1997). This may indicate a low intake of fish in leopard seals, unlike the other seals that may be preying on fish more heavily.

When the FAs of the leopard seals were compared with those of their potential prey species, there was no definitive indication that the seals were feeding on any of the prey items included in this study, irrespective of the blubber sampling site (inner or outer blubber layer). This study supports others (Best et al., 2003; Grahl-Nielsen et al., 2011; Waugh et al., 2012a) where FAs of the predator have not matched exactly with those of their prey. A close alignment between predator and prey FAs was not anticipated here as the FA results from prey were not collected at the same sampling site but prey FAs were obtained from the literature, an approach typical of other FA dietary studies (Bradshaw et al., 2003; Newland et al., 2009). The aim of this study was to identify how the inner and outer layers performed, relative to one another, in predicting dietary items. Whereas the FAs of the outer layer were clustered together and were not closely aligned with the FAs of any of the potential prey species, the FAs of the inner blubber layer aligned more closely with FAs of krill, but only on the PC2 axis (Fig. 2.6), which was driven by higher amounts of 18:4ω3 and 14:0, which are FAs from dietary origin and partially dietary, respectively (Iverson, 1993; Raclot et al., 1998). Leopard seals in this region are known to feed on krill, from scat analysis (Casaux et al., 2009), conferring with the prediction from the FA values of the innermost layer.

The ratio of vaccenic acid to oleic acid is used frequently to estimate the degree of carnivory (lower values) versus herbivory (Stübing and Hagen, 2003). The analysis of vaccenic acid/oleic acid and EPA/DHA ratios shows that the leopard seal's inner and outer blubber layers align together along with other Antarctic predators: most fish species and penguins (Fig. 2.7). The wide range of krill EPA/DHA ratios indicates that different krill species derive energy from either a diet of diatom (high ratio) or flagellate (low ratio) origin (Phleger et al., 2002). The similar trophic level of leopard seals, fish and penguins suggests that there is not a predator-prey relationship. Krill species are in lower trophic levels, which can be indicative of leopard seals preying on them. Therefore, FAs of either the inner or outer blubber layer were equally valid trophic predictors for the leopard seal.

2.7 Conclusions

Investigation of feeding ecology through FA analysis has become a valuable tool to study feeding behaviour of animals living in remote regions, like the Antarctic, where year-round studies are difficult. This study provides the first description of FA stratification of the leopard seal. As shown here, the considerable variation in FA composition across the blubber structure of the leopard seal needs to be taken into account for dietary analysis. The pattern of FA stratification is ubiquitous in marine mammals, including the leopard seal, where MUFAs are more abundant in the outer layer and PUFAs and SFAs are more abundant in the inner layer. This distribution of FAs indicates that MUFAs benefit the functional role of the outer layer as they keep the tissues flexible. Abundance of PUFAs, of dietary origin, and SFAs, good energy sources, in the inner layer supports the idea that this section of blubber has a dietary function.

The influence of diet in the outer layer is reduced since the inner layer resembles the FAs of the prey more closely. However, even though the inner layer will provide more accurate results on diet inference, this may not exactly indicate the prey items consumed by the predator, as predator's metabolism will influence their FA composition.

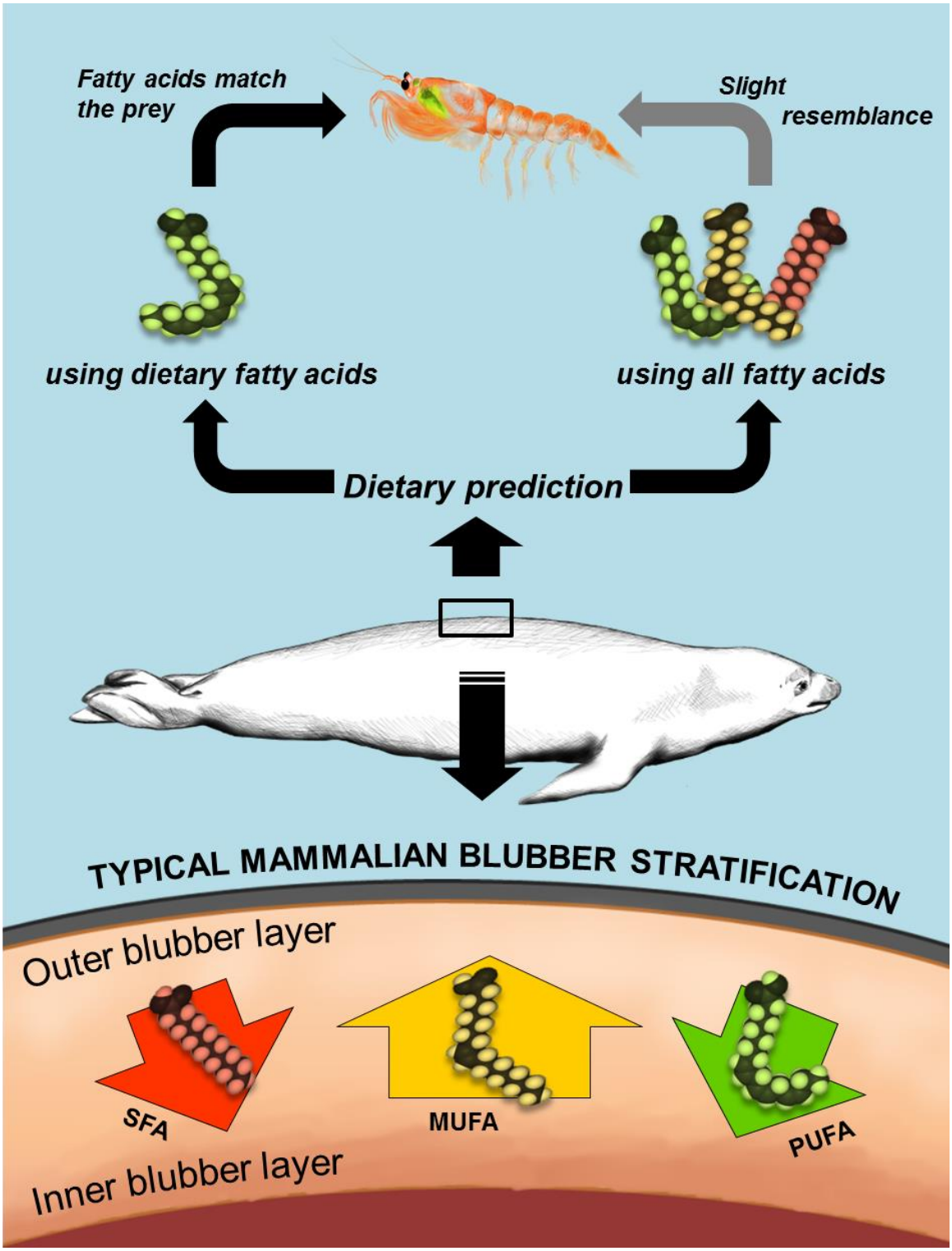
CHAPTER 3

Blubber fatty acid composition and dietary inference in crabeater seals

Part of this chapter has been previously published (see Appendix 2) in the *Journal of Experimental Biology and Marine Ecology,* as:

Guerrero, A. I., Rogers, T.L., 2017. Blubber fatty acid composition and stratification in the crabeater seal, *Lobodon carcinophaga*. J. Exp. Mar. Biol. Ecol. 491: 51-57

3.1 Graphical Abstract



3.2 Abstract

The aim of this study is to determine the FA composition across the blubber layer of the crabeater seal and validate the use of FAs as trophic markers. Seals were captured in the western Antarctic Peninsula during the austral summer of 2015 and a whole blubber core sample was collected. The blubber core was sub-sectioned into inner and outer layers and analysed for FAs. Some of the prey species were collected in the same study area and analysed for FAs, whereas for others, their FA composition was obtained from the literature. The FA composition of crabeater seals was similar to other marine mammals, with high proportions of 18:1ω9, 16:1ω7, and 16:0. These FAs are endogenous, that is, they can be readily synthesised by mammals. However, the high proportions of 22:6ω3 and 20:5ω3 are unusual among other marine mammals. These FAs are potentially reflecting the diet of crabeater seals, as they have a dietary origin and are known to be abundant in krill, the main prey item of this predator. Inner and outer layers were significantly different indicating a stratification of blubber FAs. The pattern of stratification is ubiquitous in marine mammals, with monounsaturated FAs increasing towards the outer layer, and saturated and polyunsaturated FAs increasing towards the inner layer. This difference between inner and outer layers may indicate that they have different metabolic roles. Because most dietary FAs were more abundant in the inner layer, this is likely to be the section where the deposition of FAs obtained from the diet occurs. The higher proportions of monounsaturated FAs in the outer layer suggest that this section of the blubber plays a more functional role, as these FAs improve fluidity of tissues. When compared to potential prey FAs, the blubber of crabeater seals was a good indicator of diet, particularly when using only dietary FAs in the analysis. Outer and inner layers provided the same dietary predictions when analysed including only dietary FAs.

3.3 Introduction

Due to the variety of roles played by the blubber layer, the study of its biochemical composition, specifically the FA signature, can provide insights into several aspects of the life of an animal. Consequentely, this method has become scientifically atractive and is particularly useful to study more cryptic mammals, or where the collection of samples is difficult to carry out during the whole year.

As shown previously in Chapter 2, one of the most important applications of the study of blubber FA composition is the inference of food items in the diet of a predator. However, FAs have been used to study other aspects of the physiology of marine mammals. The effect of environmental temperature on FA composition of tissues has been demonstrated in fish (Hazel, 1979; Tiku et al., 1996), and there is some evidence that suggests that a similar effect may occur in the blubber of marine mammals. For example, Samuel and Worthy (2004) found differences in FAs of bottlenose dolphins between winter and summer that can be attributed to environmental conditions. Therefore, although this area of study has been explored to a lesser extent than dietary applications, it can potentially provide valuable information on the capacity of marine mammals to adapt to different climatic conditions.

Other less explored uses of FA analysis in marine mammals include the relationships between FAs and age (Beck et al., 2007; Herman et al., 2009; Koopman et al., 1996), reproductive state (Kellar et al., 2006; Samuel and Worthy, 2004), sex (Beck et al., 2007),and diving behaviour (Koopman, 2007). FA analysis is a promising area of study that can have multiple applications when investigating different aspects of marine mammals' life history.

Here, I examine the blubber FA composition and stratification of crabetaer seals. This mammal is the most abundant phocid seal in the world (Siniff, 1991) but one of the least

studied of the Antarctic ecosystem (Casaux et al., 2009; Gales et al., 2004). They are year round residents of the antarctic pack ice (Laws, 1985), where they usually aggregate in large groups (Gales et al., 2004; Laws, 1985). Crabeater seals have lobed teeth (Siniff, 1991) that are specialised for consumption of Antarctic krill, which is their main prey item (Forcada et al., 2012; Hückstädt et al., 2012; Laws, 1985). More than 3 million crabeater seals consume over 12 million tonnes of krill each year, approximately 17% of the krill standing stock (Forcada et al., 2012).

Their blubber reaches its maximum thickness (~5 cm) in late summer/early autumn and maitains this level throughout the winter (Laws et al., 2003; McDonald et al., 2008; Øritsland, 1970b). After the pupping and breeding season that takes place in spring (Siniff et al., 1979) lipid reserves are utilised causing a reduced blubber thickness (McDonald et al., 2008; Siniff, 1991) which reaches its minimum (~2-3 cm) during early summer (Castellini et al., 2009; Laws et al., 2003). In this study, seals were captured after the breeding season in mid-summer, where seals are expected to be in a positive energy state and acculumating lipids in their blubber.

The first aim of this study was to examine the blubber FA composition of the crabeater seal. Amongst the Antarctic phocid seals, the FA composition of blubber has been reported for southern elephant seals (Best et al., 2003); leopard seals (Guerrero et al., 2016); and female Weddell seals (Wheatley et al., 2007); but not yet for Ross seals, *Ommatophoca rossii*; nor for crabeater seals.

The second aim of this study is to determine the degree of FA stratification in the blubber of crabeater seals. Most marine mammals have been reported to have a stratified blubber (Best et al., 2003; Grahl-Nielsen et al., 2011; Guerrero et al., 2016; Quérouil et al., 2013), which implies that the outer blubber layer has a different FA composition compared to the inner layer. This feature of blubber can have implications

for dietary studies (Lambert et al., 2013); therefore, it is necessary to assess the existence of FA stratification prior to further analyses. To do this, the FA composition of crabeater seals in outer and inner blubber layers were analysed separately.

Finally, I aim to determine what dietary predictions can be obtained from the blubber FAs and whether inner and outer layers will predict the consumption of similar prey items.

3.4 Materials and methods

3.4.1 Sample collection

From mid-January to mid-February of 2015, 20 crabeater seals (11 females and 9 males, Table 3.1) were sampled off the Danco Coast, Western Antarctic Peninsula (64°09′ S 60°57′ W). Immobilisation of seals, sample collection and sample preparation were performed as described in Chapter 2, section 2.4.1. Blubber cores were measured with a calliper and divided into three sections. The middle layer was not used in this analysis, as it corresponds to a transition section between outer and inner layers (See Chapter 2). This work, including the sample collection of the following chapters, was approved by the Dirección Nacional del Antártico, Buenos Aires, Argentina; and performed according to the SCAR Code of Conduct for Animal Experiments under UNSW Animal Care and Ethics Committee (Protocol 15/55A).

Krill samples were obtained in 2015 from Gentoo and chinstrap penguin stomachs. Penguins were captured in the study area when they were returning from feeding. Stomach content was obtained using the pumping method described in Wilson (1984). Other set of krill samples was obtained from the stomach of a deceased leopard seal in 2008. Although stomach acid could have potentially influenced krill FAs, samples from

both penguins and the seal were visually not degraded. Notothenid fish, *Notothenia coriicep,* were caught by angling in the same study area in summer of 2012.

Other FA data were collated from published literature, this included Antarctic krill *Euphausia superba* (Cripps et al., 1999; Phleger et al., 2002; Polito et al., 2012); the notothenid *Pleurogramma antarctica* (Mayzaud et al., 2011); the myctophids *Electrona carlsbergi*, *Gymnoscopelus nicholsi*, *Gymnoscopelus piabilis*, *Protomyctophum bolini*, *Lampichthys procerus,* and *Metelectrona ventralis* (Connan et al., 2010; Raclot et al., 1998); and the squid *Moroteuthis ingens* (Raclot et al., 1998).

Table 3.1. Parameters measured in the 20 crabeater seals sampled.

Seal ID	Sex	Age Class	Body Mass (Kg)	Blubber Thickness (cm)	Body Length (m)
CS1501	Male	Sub-adult	226	2.5	2.13
CS1502	Male	Adult	246.4	2.6	2.27
CS1503	Male	Adult	259.6	2.6	2.20
CS1504	Female	Adult	-	3.4	2.49
CS1505	Female	Sub-adult	199	3.0	2.00
CS1507	Male	Sub-adult	196.6	2.3	1.93
CS1508	Male	Adult	271.4	2.2	2.25
CS1509	Male	Adult	278.6	2.5	2.38
CS1510	Female	Adult	266.6	1.4	2.38
CS1511	Female	Sub-adult	216.6	2.6	2.06
CS1512	Male	Adult	260	1.8	2.32
CS1513	Female	Adult	266.2	1.6	2.25
CS1514	Female	Adult	269	2.1	2.40
CS1515	Female	Sub-adult	159.8	3.2	1.89
CS1516	Male	Adult	226.6	3.1	2.17
CS1517	Male	Adult	271.6	2.3	2.32
CS1518	Female	Sub-adult	146.4	1.8	1.82
CS1519	Female	Adult	267.4	3.0	2.24
CS1520	Female	Sub-adult	163.6	3.6	1.86
CS1521	Male	Adult	240.4	2.8	2.23

3.4.2. Fatty acid analysis

Total lipid of crabeater seal blubber and whole krill samples was extracted following a modified Folch et al. (1957) method (Budge et al., 2006). Briefly, samples were extracted with 2:1 chloroform: methanol containing 0.01% of butylated hidroxytoluene, washed in a salt solution, centrifuged, dried over anhydrous sodium sulphate and evaporated under nitrogen.

Fatty acid methyl esters (FAMEs) were prepared with an acidic transesterification procedure, using H_2SO_4 in methanol (Budge et al., 2006). Esters were extracted into hexane at a concentration of 50 mg/mL and stored in vial tubes for gas chromatography analysis.

Gas chromatography analyses were performed with Agilent 7890A Series GC System (Agilent Technologies, U.S.A.) equipped with a flame ionization detector. FAMEs were analysed using a 30 m x 0.25 mm flexible fused silica column coated with 50% cyanopropyl polysiloxane (0.25 μm film thickness, DB-23, Agilent Technologies, U.S.A.). The injector and detector port temperatures were held at a constant temperature of 250°C. The helium split flow was set at a rate of 100 mL/min and the carrier gas flow (He) was 1 mL/min. The flow rates of air and hydrogen to the detector were 450 mL/min and 45 mL/min, respectively. The column oven was initially held at 153°C for 2 min, and then increased at a rate of 2.3°C/min to 174°C. That temperature was maintained for 0.2 min and then ramped at 2.5°C to 210°C. This final temperature was held for 2 min. Thus, the total run time per cycle was 28 minutes. Peak areas and retention times were calculated (ChemStation Software, Rev. B.03.01; Agilent Technologies), and FAMEs were identified by comparison of retention times with a range of standards. The concentrations of individual FAs in each sample were converted to percentage contributions of the total FAs.

3.4.3 Data analyses

Only FAs present in amounts >0.5% were included in these analyses, since trace amounts are more likely to contribute noise (Grahl-Nielsen et al., 2011). Thus, the number of FAs was reduced to 17. Due to the multivariate nature of FA data, Principal Component Analysis (PCA) was used to determine patterns in the FA composition of crabeater seals. In this manner the 17 variables corresponding to each FA identified could be described in two dimensions. Due to proportional FA data does not have a normal distribution, this was log transformed according to the formula recommended by Aitchison (1986), using 18:0 as reference as suggested by Budge et al. (2006). Multivariate analysis of variance (MANOVA) was then applied to the PC scores to test for differences between blubber layers, age classes and sexes.

To further investigate the patterns in stratification of FAs, paired sample t-tests were applied to test for differences in FA types (saturated FAs, SFAs; monounsaturated FAs, MUFAs; and polyunsaturated FAs, PUFAs) between outer and inner layers. In addition, a stratification index (SI) was calculated according to the equation by Olsen and Grahl-Nielsen (2003) described in Chapter 2, section 2.4.3.

Simple linear regressions were used to test whether FA stratification index was correlated with body mass or blubber thickness.

For dietary inference, FA values were log-transformed to level out the quantitative differences among FAs. A PCA was computed in order to describe the relationships between prey species and predator samples, projected in two dimensions. I computed two different PCA, one using all 17 FAs and one using only 8 dietary FAs present in both prey and predator, which were the MUFA 22:1ω11 and the PUFAs 18:2ω6, 18:3ω3, 18:4ω3, 22:2ω6, 20:5ω3, 22:4ω3 and 22:6ω3. In order to determine how inner and outer layers perform relative to each other as dietary predictors, I performed another

PCA analysis using only dietary FAs and including either only the inner or only the outer blubber layer. I then used the first three PC scores of each analysis and computed a quantitative measure for the difference between the samples, using a cluster analysis of Euclidean distances.

Additionally, I performed a discriminant function analysis (DFA) using the first three components derived from the PCA that included only dietary FAs from the inner layer. I used prey samples as the training set and seal blubber samples as the test set. Thus, the analysis predicted the group membership of the seal samples for prey type (fish, krill or squid).

All statistical tests were performed in R (Team, 2016) and significance was deemed with an $\alpha < 0.05$ or when confidence intervals (CIs) extracted from slope values did not overlap 0. As for Chapter 2, values are reported as mean ± standard deviation (SD), unless otherwise noted.

3.5 Results

3.5.1 Fatty acid composition

Blubber cores had a thickness of 2.52 ± 0.60 cm (Table 3.1). Only 17 FAs were found in amounts greater than 0.5% across all blubber samples (Table 3.2). The most abundant FAs, in decreasing order, were: 18:1ω9, 16:1ω7, 22:6ω3, 20:5ω3, 16:0, 18:1ω7 and 14:0, which accounted for approximately 75% of total FAs (Fig. 3.1).

Overall, the most abundant type of FA in the blubber of crabeater seals was MUFA (Table 3.2, Fig. 3.2), which comprised 40.95 ± 4.56% of total FA in the inner layer, and 53.31 ± 3.42% in the outer blubber layer. The proportion of MUFAs was significantly different between layers (t_{19} = 9.782, P < 0.001). PUFAs were the second most

abundant type of FA, being significantly (t_{19} = -8.947, P < 0.001) more abundant in the inner layer with 35.07 ± 2.74% compared to the outer layer with 26.16 ± 3.95%. Similarly, SFAs were significantly (t_{19} = -7.008, P < 0.001) more abundant in the inner than in the outer layer, with 17.03 ± 2.37% and 12.67 ± 1.99% respectively.

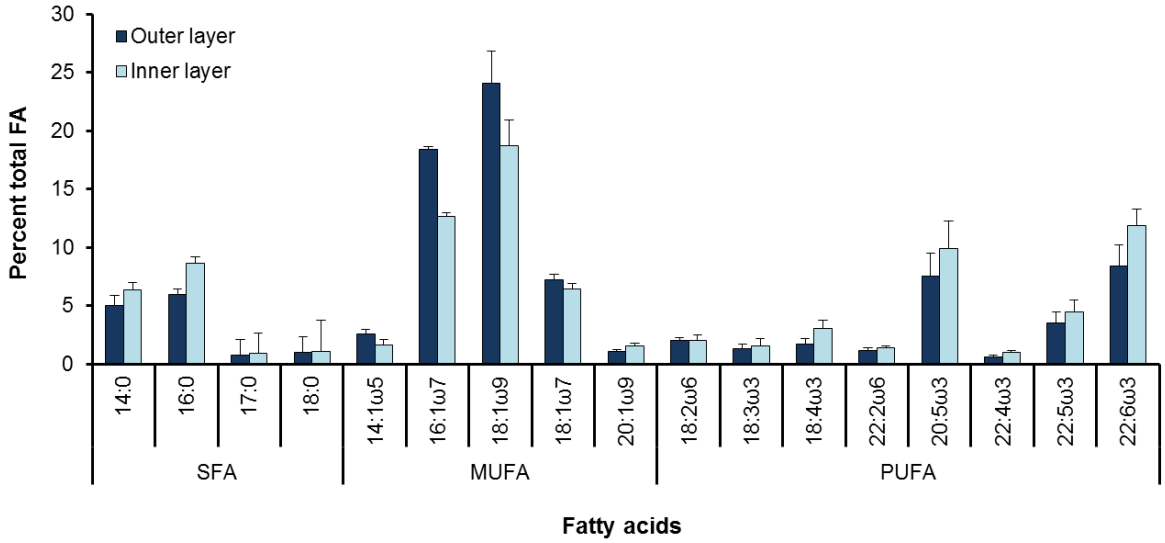


Fig. 3.1. Proportion of FAs in the blubber of crabeater seals. Per cent total of 17 FAs in the inner and outer blubber layers of 20 crabeater seals.

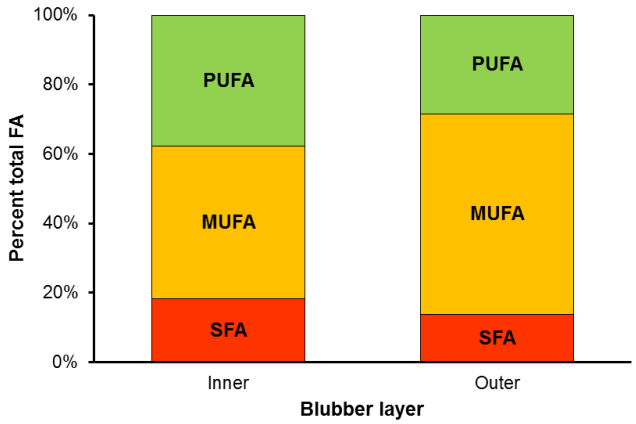


Fig. 3.2. Fatty acid types in inner and outer blubber layers. Mean proportion of polyunsaturated (PUFA), monounsaturated (MUFA) and saturated (SFA) fatty acids for crabeater seals (n=20).

Table 3.2. Fatty acid composition* (per cent total FA + SD) of the inner and outer blubber layers of crabeater seals (n = 20).

	Inner Layer			**Outer Layer**		
	Mean		SD	Mean		SD
14:0	6.40	±	0.60	5.00	±	0.85
16:0	8.66	±	1.83	5.93	±	1.31
17:0	0.90	±	0.47	0.76	±	0.39
18:0	1.07	±	0.27	0.97	±	0.23
Σ SFA	**17.03**	**±**	**2.37**	**12.67**	**±**	**1.99**
14:1ω5	1.62	±	0.58	2.56	±	0.47
16:1ω7	12.50	±	2.66	18.40	±	1.34
18:1ω9	18.84	±	2.22	24.05	±	2.80
18:1ω7	6.41	±	0.43	7.21	±	0.48
20:1ω9	1.57	±	0.45	1.09	±	0.27
Σ MUFA	**40.95**	**±**	**4.56**	**53.31**	**±**	**3.42**
18:2ω6c	2.01	±	0.25	1.98	±	0.17
18:3ω3	3.08	±	0.74	1.68	±	0.48
18:4ω3	1.56	±	0.62	1.31	±	0.39
22:2ω6	1.37	±	0.21	1.18	±	0.24
20:5ω3	9.81	±	2.29	7.50	±	1.98
22:4ω3	0.97	±	0.19	0.59	±	0.17
22:5ω3	4.44	±	1.04	3.52	±	0.92
22:6ω3	11.83	±	1.52	8.41	±	1.80
Σ PUFA	**35.07**	**±**	**2.74**	**26.16**	**±**	**3.95**

* Only those FAs contributing >0.5% of the total are shown

3.5.2 Fatty acid stratification

The stratification index indicates the inner-outer differences per individual FA (Fig. 3.3). A negative stratification index was found in all SFAs and PUFAs, indicating their greater abundance in the inner layer. Most MUFAs had a positive stratification index due to their greater dominance in the outer layer. The only MUFA with a negative index was the long-chain 20:1ω9, which was enriched in the inner layer. The mean overall stratification index was 0.27 ± 0.16 and the most stratified FA was 18:4ω3, with an index of -0.58. Although there was a slight positive relationship, the stratification index was not significantly correlated with either body mass (slope = 0.008; CIs = -0.0004, 0.0019) or blubber thickness (slope = 0.0051; CIs = -0.0034, 0.0136).

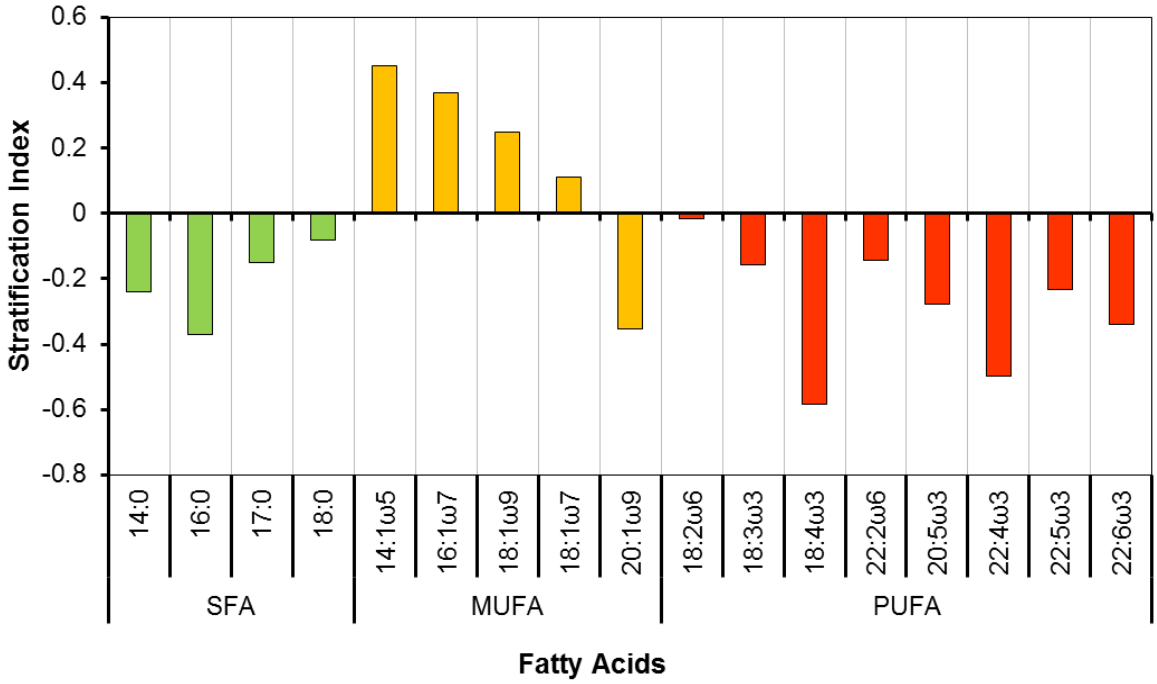


Fig. 3.3. Stratification index for FAs in inner and outer blubber layers of crabeater seals (n = 20). Positive values indicate a greater proportion of the fatty acid in the outer layer and negative values indicate a greater abundance in the inner layer.

Principal component analysis, including all 17 individual FAs, confirmed separation between blubber layers. Figure 3.4 shows inner and outer layers as separated groups. PC1 explained 46% of the variation while PC2 explained 25%. The separation of blubber layers was evident on the PC2, where the negative Eigenvalues of 18:4ω3 and 22:4ω3 and positive Eigenvalues of 14:1 and 16:1 drove most of this segregation. A MANOVA applied to the first 2 PC scores confirmed that inner and outer layers were significantly different (*Wilks' λ* = 0.381, $P < 0.001$). However, there were no significant differences between sexes (*Wilks' λ* = 0.976, $P = 0.683$), or age classes (*Wilks' λ* = 0.973, $P = 0.664$), or interaction between any of these variables.

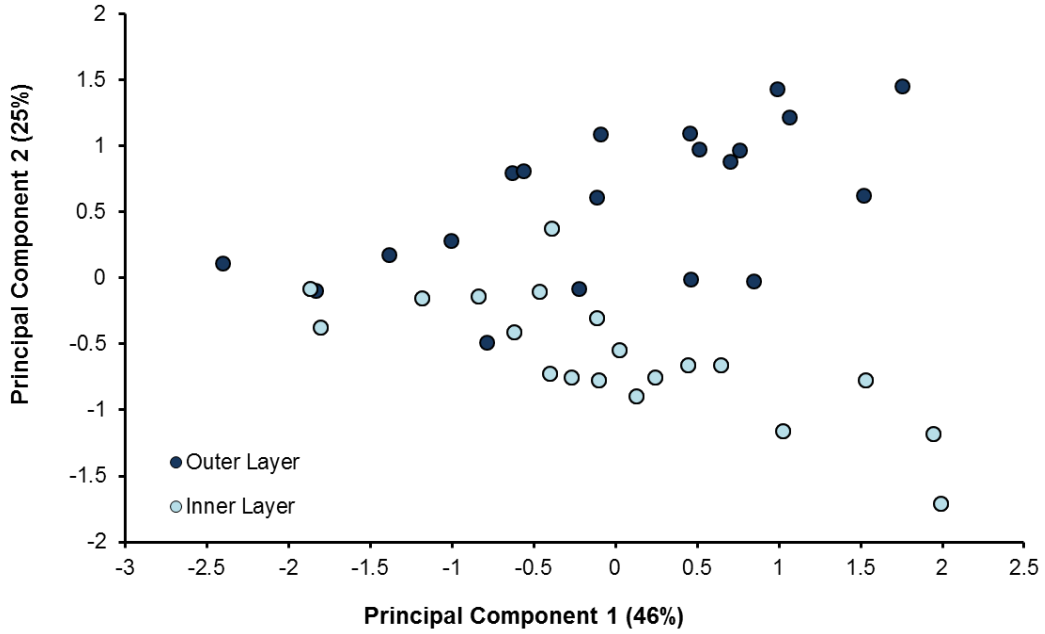


Fig. 3.4. Stratification of FAs across crabeater seal blubber. Principal component (PC) plot for FAs in the inner and outer blubber layers of crabeater seals (n=20).

3.5.3 Dietary predictions

The PC plot including all 17 FAs (Fig. 3.5A) shows no distinguishable difference between inner and outer layers. PC1, which explained 66% of the variation, is segregating prey species and crabeater seals. The smallest difference was between two samples of krill (from the same study area) and the crabeater seals species. PC2 explained 18% of the variation and was mostly separating krill from fish species. Figure 3.5B, which was plotted using only dietary FAs, shows a clearer resemblance between some of the krill samples and crabeater seals. Once again, inner and outer blubber layers occupied the same position in the plot area.

Cluster analysis on the first three PCs confirmed the resemblance between the crabeater seals and krill samples from the same study area (Fig. 3.6). The prediction obtained using only the inner layer (Fig. 3.6A) was the same compared to using only the outer

blubber layer (Fig. 3.6B), which indicated that the smallest Euclidean distance was between the crabeater seals (inner or outer layer) and the two krill samples obtained in the same study area (K1 and K2).

The calculation of the DFA containing prey samples as the training set produced a correct classification of 80% of prey species, where 1 krill species was classified as fish, and 2 fish species were classified as krill. Using the DFA for seal blubber samples as the test set, all 20 crabeater seals were identified as belonging to the krill group (Fig. 3.7).

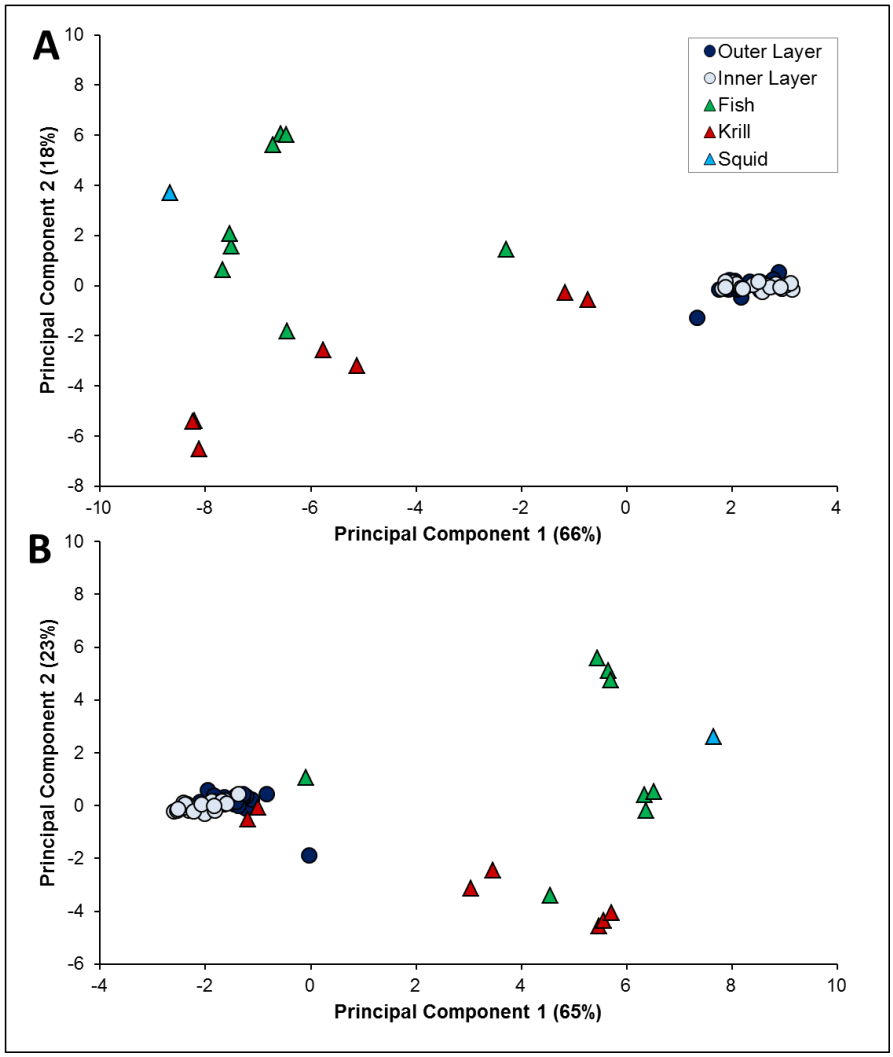


Figure 3.5. Principal component plot for samples of inner and outer blubber layers of crabeater seals and their potential prey species, including krill, fish and squid. Plot A) is the outcome using all 17 FAs and plot B) includes only 8 dietary FAs.

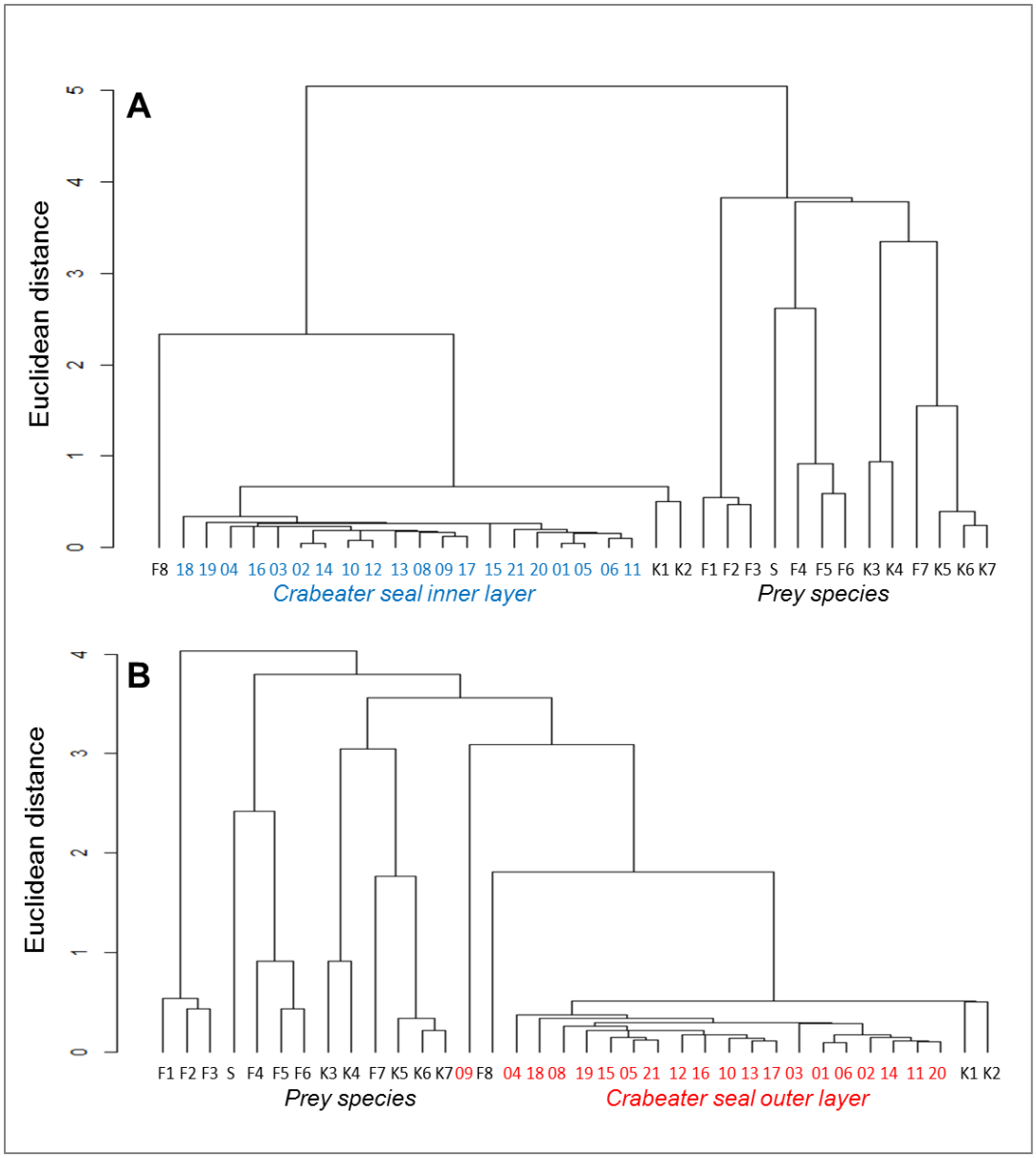


Figure 3.6. Dendogram of the Euclidean distances between the PC scores of crabeater seals and potential prey spcecies. Diagram A) is based on the PC scores of the inner blubber layer of crabeater seals, whereas B) corresponds to the PC scores of the outer blubber layer. The prey types used are: fish (F1 = *M. ventralis*, F2 = *G. piabilis*, F3 = *L. procerus*, F4 = *P. bolini*, F5 = *E. carlsbergi*, F6 = *G. nicholsi* , F7 = *P. antarcticum*, F8 = *N. coriiceps*), krill, *E. superba* (K1 and K2 are krill from the same study area from 2008 and 2015, respectively; K2 to K7 were obtained from the literature and correspond to different years and locations within the Southern Ocean) and a squid (S = *M. ingens*).

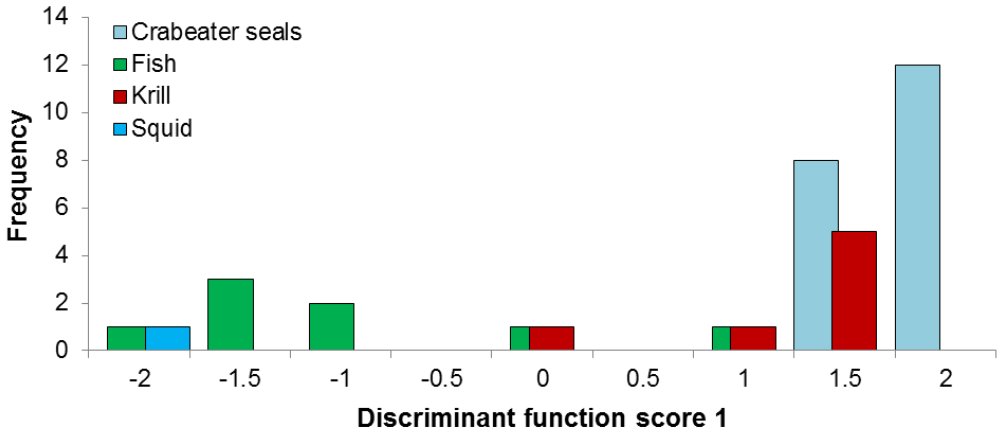


Figure 3.7. Histogram of linear discriminant scores derived from prey data. Based on these scores, all 20 crabeater seals were classified into the krill group.

3.6 Discussion

3.6.1 Fatty acid composition of blubber

Crabeater seals possess a FA composition similar to other marine mammal top predators. Large proportions of 18:1ω9, 16:1ω7, 16:0 and 18:1ω7 have been reported for other phocids, including Weddell seals (Wheatley et al., 2007); leopard seals (Guerrero et al., 2016); southern elephant seals (Best et al., 2003); and ringed seals (Strandberg et al., 2008); and for cetaceans including dusky dolphins (Grahl-Nielsen et al., 2010); Atlantic spotted dolphins, *Stenella frontalis* (Quérouil et al., 2013); white whales, *Delphinapterus leucas* (Dahl et al., 2000); fin whales (Ruchonnet et al., 2006). These four FAs have been identified as endogenous, indicating that they can be readily synthesised by the animal (Herman et al., 2005; Iverson, 1993); therefore they should not be influenced by the type of prey consumed by the predator. In fact, most of these FAs are not only ubiquitous to marine mammals but are also abundant in terrestrial mammals. For example, the FA 18:1ω9 comprises about 57% of the fat of the 13-lined

ground squirrel (Price et al., 2013), and 43% in the brown bear, *Ursus arctos* (Käkëla and Hyvärinen, 1996a).

The FA 16:0 is generally more abundant in terrestrial than in marine mammals, with values higher than 20% in domestic pigs, *Sus scrofa domesticus* (Apple et al., 2009; Mackay et al., 2013); grey wolfs, *Canis lupus*; brown bears; and raccoon dogs, *Nyctereutes procyonoides* (Käkëla and Hyvärinen, 1996a); whereas the values of 16:0 in marine mammals fluctuate between ~4% in bottlenose whales, *Hyperoodon ampullatus* (Hooker et al., 2001), and ~15% in New Zealand sea lions (Lambert et al., 2013) with the exception of manatees, *Trichechus manatus* (Ames et al., 2002), which have large amounts of 16:0 (~26%) compared to their marine counterparts.

Another abundant FA in crabeater seals is 14:0. This is a partially endogenous FA, which can be biosynthesised by the animal but can also have a dietary origin (Iverson, 1993). The value of crabeater seals is generally similar to values found in other marine mammals (eg. Arriola et al., 2013; Guerrero et al., 2016; Thiemann et al., 2008)

The other two FAs found in high proportions in crabeater seals are 22:6ω3 (DHA) and 20:5ω3 (EPA). These FAs originate from the diet (Iverson, 1993; Raclot et al., 1998) and are particularly abundant in krill and other pelagic crustaceans (Phleger et al., 2002). Similar high values of DHA and EPA have been found in arctic seals, including ringed seals (Grahl-Nielsen et al., 2005; Käkëla et al., 1993; Strandberg et al., 2008), ribbon seals, *Phoca fasciata* (West et al., 1979a); and bearded seals, *Erignathus barbatus* (West et al., 1979a). In Antarctic seals, on the other hand, DHA and EPA are not very abundant. Weddell and southern elephant seals have ~5% of DHA and ~3% of EPA (Best et al., 2003; Wheatley et al., 2007) whereas leopard seals have ~6% and ~5%, respectively, which may be due to leopard seals preying on krill (Guerrero et al., 2016). Humpback whales, *Megaptera novaeangliae*, who are also krill consumers, have

reported ~6% of DHA and ~8% of EPA in their outer layer (Waugh et al., 2012b); because of their dietary origin, these values are expected to be higher in the inner layer. Crabeater seals are krill specialists (Hückstädt et al., 2012); therefore their values of ~10% of DHA and ~12% of EPA in the inner blubber layer would indicate the dietary influence in their FA composition.

Conversely, high levels of 20:1ω9 and 22:1ω11 tend to be characteristic of many teleost fish (Iverson et al., 1997); therefore they are expected to be more abundant in fish consumers. Crabeater seals have ~1.5% of 20: 1ω9, which is low compared to southern elephant seals (Best et al., 2003); harp seals (Tucker et al., 2009); and hooded seals, *Cystophora cristata* (Tucker et al., 2009); with ~15% of 20: 1ω9. The other FA, 22:1ω11, was only present in trace amounts in crabeater seals. This could indicate that crabeater seals are not incorporating large proportions of fish in their diet.

3.6.2 Stratification of fatty acids: the roles of inner and outer layers

Stratification implies that FAs are being deposited/mobilised differentially through the depth of the blubber core (Koopman, 2007). The identification of stratification in marine mammal blubber is important for dietary studies. When outer and inner blubber layers are substantially different, the use of the inner layer is recommended for dietary prediction (Grahl-Nielsen et al., 2005; Koopman, 2007; Olsen and Grahl-Nielsen, 2003).

Early studies in the biochemical composition of whale blubber suggested that the inner layer was more metabolically active and had a role related to the dietary process (Ackman et al., 1965; Lockyer et al., 1984). Similarly, undernourished harbour porpoises and bottlenose dolphins have been found to reduce the number and size of adipocytes (lipid cells) only in the inner blubber layer whereas the outer layer remains

stable (Koopman et al., 2002; Struntz et al., 2004). This indicates that the dietary process of depletion and accumulation of lipids occurs in the innermost section of the blubber layer (Struntz et al., 2004).

The higher proportion of PUFAs found in the inner layer of crabeater seals supports this idea (Fig. 3.2). These FAs originate in lower trophic levels and they move up the food web as they are consumed by consecutively higher level predators (Hoberecht, 2006). Because mammals do not have the ability to synthesise PUFAs (Iverson, 1993), the higher presence of these FAs in the inner layer suggests that the deposition/mobilisation of dietary FAs takes place here.

The higher proportion of SFAs in the inner layer also supports the idea of its dietary role. These FAs offer more chemical energy per unit mass (Maillet and Weber, 2006); therefore they can be used as an energy resource when needed.

The pattern of stratification seen in crabeater seals was similar to that found in most marine mammals (eg. Arnould et al., 2005; Grahl-Nielsen et al., 2005; Lockyer et al., 1984; Meier et al., 2016), with MUFAs more abundant in the outer compared to the inner layer, and SFAs with the opposite tendency (Fig. 3.2). This suggests that the drivers behind the FA stratification in marine mammals are the same in all species (Olsen and Grahl-Nielsen, 2003). One explanation to the higher level of desaturation (higher presence of MUFAs) in the outer layer, is that these FAs are not readily mobilised by the animal; therefore they accumulate in this blubber layer (Koopman et al., 1996). Another explanation is the potentially thermoregulatory role of the outer layer (Grahl-Nielsen et al., 2005). A higher proportion of MUFAs in the outer blubber layer would lower its solidifying point, making the tissues less rigid in cold temperatures (Irving and Hart, 1957). Since most MUFAs and SFAs are endogenous FAs (Iverson, 1993), animals have the ability to synthesise higher amounts of MUFAs,

modifying their saturated counterparts (SFAs). For example, the SFA C16:0 may not be abundant in the outer layer, since it has been turned into the MUFA 16:1ω7, because the latter withstands lower temperatures without solidifying. Thus, the inner layer has higher proportions of SFAs, which are solid at room temperature (Christie, 2003), but in the warm inner layer that is not a problem. However, in the cold outer layer, higher amounts of SFAs would make the tissue very rigid, therefore the transformation of SFAs into MUFAs, or desaturation of FAs, is necessary.

While this pattern of stratification appears to be universal, the degree of stratification varies among marine mammals. Koopman (2007) found increased stratification in odontocetes inhabiting colder waters, compared to species from warmer habitats, suggesting that the thermal regime is a significant factor shaping the structure of the blubber. Additionally, studies on the thermal properties of the blubber of three cetacean species have reported that the outer layer have lower thermal flux values compared to the inner layer (Bagge et al., 2012; Dunkin et al., 2005). Therefore, Bagge et al. (2012) suggests that the function of blubber as insulator may depend on its stratification and the different heat-storage capabilities of outer and inner layers. These findings suggest that crabeater seals, which are inhabitants of an extremely cold environment, should have a high degree of FA stratification compared to phocids from warmer regions.

Koopman (2007) states that the blubber of odontocetes may be more stratified than that of most other marine mammals. For example, harbour porpoises have a stratification index of 0.79 (calculated from Koopman et al., 1996) and dusky dolphins of 0.81 (calculated from Grahl-Nielsen et al., 2010). On the other hand, otariids possess a blubber layer with the lowest values of stratification, including cape fur seals with 0.10 and New Zealand sea lions with 0.14 (Arnould et al., 2005; Lambert et al., 2013). In phocids, the difference between outer and inner layers is more pronounced than in

otariids (Lambert et al., 2013), which also supports the idea of the thermoregulatory role of a highly stratified blubber, since otariids in general inhabit warmer environments than phocids.

However, this does not explain the high stratification values of odontocetes, as they also inhabit temperate waters. The variation in stratification is complex and likely related to other factors such as taxonomy, blubber thickness and body size (Koopman, 2007; Lambert et al., 2013).

Compared to their Antarctic counterparts, the overall FA stratification index of crabeater seals (0.27 ± 0.16) is lower than that of leopard seals (0.44 ± 0.22) from the same region in western Antarctica (Guerrero et al., 2016), but higher than that of Weddell seals (0.23) from eastern Antarctica (calculated from Wheatley et al., 2007). Although these three species inhabit similar climatic regions and therefore their stratification values are expected to be similar, the stratification index may be affected by other factors. Weddell seals in the mentioned study were sampled post-partum (Wheatley et al., 2007), which means they were probably using the lipids stored in the inner layer, which could account for the low values of stratification. In the case of crabeater seals, it is very likely that these animals had not completely recovered the inner layer utilised during the spring breeding season (Southwell et al., 2003); as samples were taken in mid-summer and they reach a maximum thickness towards the end of summer or beginning of autumn (McDonald et al., 2008); therefore the differences between inner and outer layer could have been underestimated.

Additionally, if the synthesis of MUFAs in the outer layer is a consequence of low temperatures, animals with thinner blubber layers could be expected to have more similar inner and outer layers, since cold temperatures could affect both layers. However, in this study the stratification of blubber FAs did not show any association

with blubber thickness, which coincides with what was found in odontocetes by Koopman (2007). The density of fur could also impact the degree of the stratification, since this would affect the temperature at which the outer layer is maintained. Consequently, although crabeater, Weddell and leopard seals share a similar environmental temperature, it is necessary to consider other factors in order to understand their differences in the degree of blubber FA stratification.

Stratification of FAs has been explained by the influence of several roles played by this tissue (Lambert et al., 2013). There may be complex factors structuring the overall composition of the blubber and they can function differently in every species or even at an individual level. However, this aspect of the blubber composition should not be overlooked as new data could contribute to the understanding of the drivers of FA metabolism at a bigger scale.

3.6.3 Use of fatty acids for dietary inference

Although blubber FAs resemble those of the diet, the use of FA analysis for dietary inference is still under debate (Grahl-Nielsen et al., 2011; Grahl-Nielsen and Mjaavatten, 1991; Iverson et al., 2004). As a specialist top predator (Hückstädt et al., 2012), the crabeater seal is a good candidate to test whether the use of FA analysis is valid for dietary predictions, as a clearer FA signature (compared to a generalist predator) is expected. As mentioned earlier in the discussion, there are some specific FAs that can give us an idea of what the animal is preying on. In this study the array of FAs was also a good indicator of the consumption of krill by this mammal.

Since the ability of mammals to synthesise their own FAs can complicate the use of FAs for dietary analysis, I tested here whether the use of all dietary and non-dietary FAs

versus the use of only dietary FAs would result in the same dietary predictions. Figure 3.5 shows that dietary FAs can provide a clearer picture of the relationships among prey species and predator. When all FAs are used, a considerable amount of noise is included in the analysis as mammals will modify or synthesise FAs when they need to fulfil their physiological demands. Very few studies have found close resemblance between predator and prey FAs (eg. Hooker et al., 2001). Most studies have found that the inner layer never exactly matches the FAs of the prey (Best et al., 2003; Grahl-Nielsen et al., 2011). In this study I found very clear resemblance between crabeater seal and krill (Fig. 3.5B), which was confirmed by the DFA performed using dietary FAs (Fig. 3.7). This can be due to they feed almost exclusively on this prey species (Laws, 1977) and hence the FA signature is more evident.

The fact that other krill samples were not very similar to crabeater seal FAs can be attributed to the variation of FAs with location and time. It is known that FAs can change at very low trophic levels according to the geographical location (Cripps et al., 1999), season (Jeffries, 1969), thermal habitat (Guschina and Harwood, 2009), among others; and that these changes will be transferred to higher trophic levels. Therefore, the use of prey samples from the same temporal and spatial location is important for better results.

When inner and outer layers have been used for dietary studies, the inner layer is closest to the composition of the potential diet (Andersen et al., 2004; Guerrero et al., 2016; Olsen and Grahl-Nielsen, 2003). In this case, using cluster analysis, inner and outer layers predicted the same dietary items (Fig. 3.6), which indicates that the FA signature is present in both blubber layers. The reason of these similar predictions is probably the use of only dietary FAs. Although these dietary FAs are in greater amount in the inner layer, the outer layer possess the same array but in smaller quantities. Since those FAs

(particularly MUFAs) that are potentially produced to fulfil other demands have been excluded from the analysis, the confounding factor present in the outer blubber layer has been greatly reduced. Another explanation is related to the low degree of stratification on crabeater seals compared to other seals. A low stratification index indicates that inner and outer layers are not very different; therefore prey FAs can be identified in both inner and outer layers. Although the inner layer is usually recommended for dietary studies, my results indicate that in some species the use of the outer layer can be as effective as the use of the inner layer.

3.7 Conclusions

This study provides the first description of FA composition of the blubber of crabeater seals. The most abundant FAs found here are also present in great amounts in other marine mammals. However, high proportions of DHA and EPA, are not ubiquitous in other mammals and suggest the influence of diet in the blubber FAs of crabeater seals.

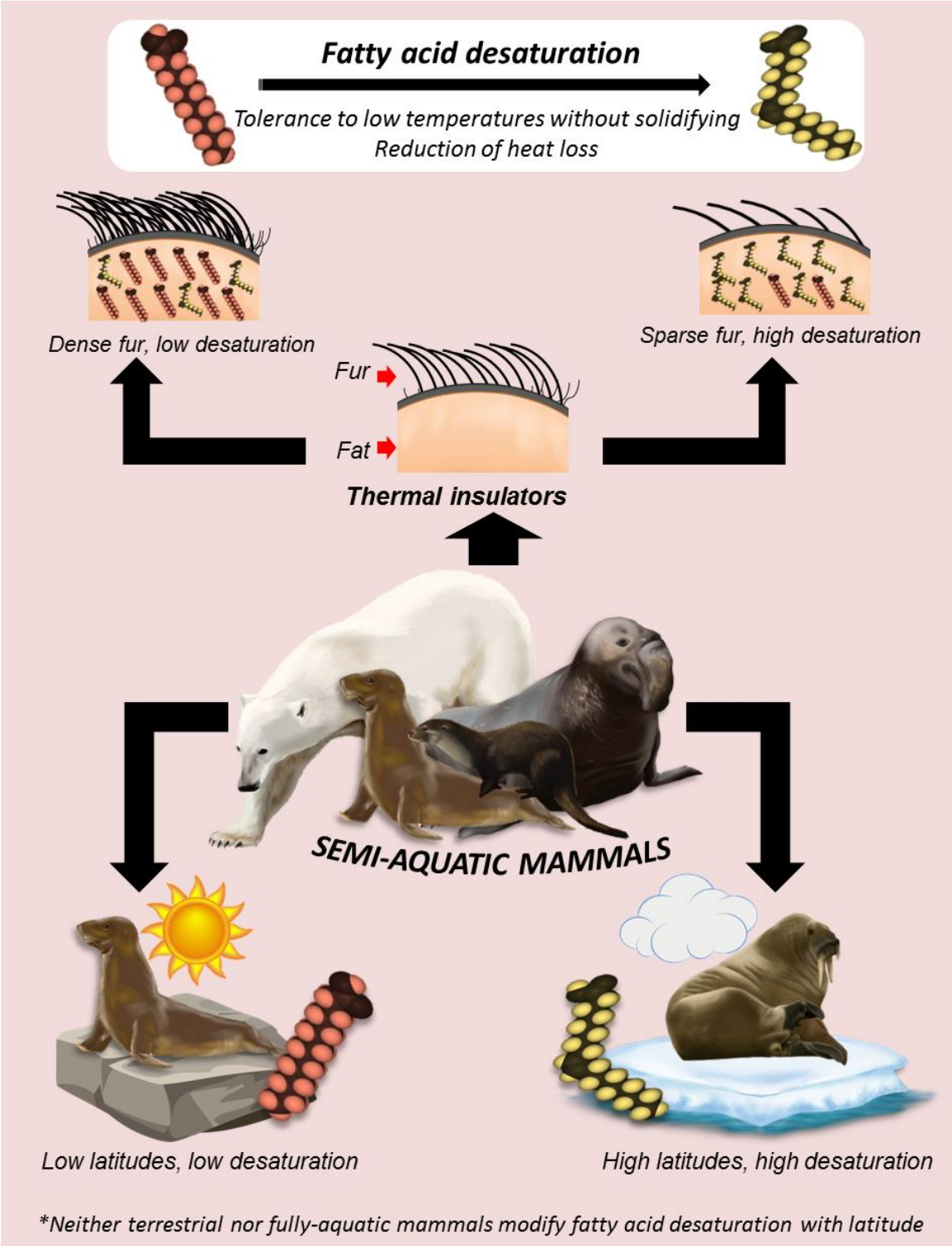
Inner and outer layers were significantly different in crabeater seals, which confirms the existence of a vertical variation, or stratification, of blubber FAs. The stratification pattern was similar to that found in most marine mammals. The higher proportion of exclusively dietary FAs in the inner layer suggests that this stratus plays a diet-related role, whereas the higher amount of MUFAs in the outer layer suggests a more functional and structural role.

Dietary predictions are more accurate using only dietary FAs. Both inner and outer blubber layers perform well predicting diet when only dietary FAs are included in the analysis.

CHAPTER 4

From land to water: the thermoregulatory role of fatty acids in the mammalian fat tissue

4.1 Graphical abstract



4.2 Abstract

The biochemical composition of adipose tissues plays an important role as a physical barrier in mammalian thermoregulation. As FA desaturation increases, adipose tissues can reach colder temperatures without solidifying. I test here whether FA composition in mammals is driven by thermal forces by investigating how variation in FA desaturation relates to different thermal proxies. I calculated a FA desaturation index for 48 species of terrestrial, semi-aquatic and fully-aquatic mammals, based mostly on data collated from the literature, and compiled a dataset of thermal proxies: hair density, latitude and environment. To examine the relative contribution thermal factors play in the variation of FA desaturation, I used phylogenetic regression analyses along with a model selection approach. An interaction of environment and latitude was the model with the highest support. Fully- and semi-aquatic mammals have higher FA desaturation compared to terrestrial mammals. Semi-aquatic mammals have significantly higher levels of desaturated FAs when living in colder environments whereas terrestrial and fully-aquatic mammals do not. I show that as mammals re-invaded aquatic environments, FA modification became an increasingly important component of their thermoregulatory strategy. A high FA desaturation allows fully-aquatic mammals to maintain pliable blubber in cold environments, but surprisingly they do not modify FAs as they migrate through different latitudes. Potentially this is because they regulate other parameters of blubber instead, such as lipid content or thickness. Semi-aquatic mammals show a compromise between fur and blubber as an insulator: when fur is very dense the desaturation of FAs is low, and vice versa. Semi-aquatic mammals in colder environments tend to rely on blubber as an insulator. Terrestrial mammals rely less on adipose tissue as an insulator, as reflected by their low FA desaturation, which does not change with latitude.

4.3 Introduction

In spite of the thermal challenges of the aquatic medium, mammals have colonised this environment on seven separate occasions and have sucessfully adapted (Uhen, 2007) . Mammals exploit a wide range of thermal habitats, from the tropics to the poles, and from high altitudes to deep oceans; therefore they possess varied mechanisms to regulate their body core temperature. Changes in body size (Meiri and Dayan, 2003), hair density (Cooper, 2003), metabolic rate (Irving and Hart, 1957), blubber thickness (Castellini et al., 2009; Pabst et al., 1999), and blubber thermal conductivity (Kvadsheim et al., 1996) are some of these mechanisms, which have been well studied. However, the importance of the biochemical composition of the adipose tissue to mammalian thermoregulatory strategy has received less attention and yet is likely to be important, particularly where mammals use blubber as a thermal barrier.

Unlike other tetrapods, within the mammalian taxa there are different insulation strategies, from fully furred through to totally naked forms, each with different thermal benefits and constraints. Where mammals do not use fur, the adipose tissue is expected to play a more important role in keeping warm. In order to reduce the heat lost to the environment, the cooling of the body surface in contact with air or water in bared-skinned animals is of critical importance. Peripheral vasoconstriction occurs in cold superficial tissues and reduces blood flow (Elsner, 1999), thus creating a thermal gradient through the adipose tissue and reducing body heat loss.

But cooling the body surface to temperatures similar to those of the ambient could create a potential problem. In extremely cold environments, low temperatures could cause rigidity and solidification of superficial tissues; it is here when the biochemical structure of fats becomes critical.

The adipose tissue, or blubber in aquatic mammals, was originally considered to be a simple storage organ for fat, but recent advances have demonstrated that this subcutaneous tissue is not a passive fat storage depot but a dynamic and complex organ (Hertzel et al., 2008). The structure of the FAs making up the adipose tissues will have different effects on the properties of this organ. The degree of desaturation of a FA influences its solidifying (or melting) point. The solidifying point describes the change between solid and liquid phases in response to temperature changes (Irving et al., 1957). Thus, polyunsaturated FAs (PUFAs) have lower solidifying points than monounsaturated (MUFAs) and saturated FAs (SFAs). As the degree of desaturation increases (i.e. when SFAs are turned into MUFAs), the tissues become more fluid and can reach colder temperatures for longer periods without solidifying (Irving et al., 1957). A blubber layer with higher degree of desaturation is therefore better suited for lower temperatures and thus more effective for the maintenance of a thermal gradient and consequently better for heat loss reduction.

If the type of FAs comprising the adipose tissues is driven by thermal conditions then I should be able to identify patterns in the composition of non-dietary FAs (those that can be readily synthesized by the animal) across mammals exposed to different thermal conditions.

The aim of this study was to evaluate potential thermal influences on non-dietary FAs in mammals. I investigated the differences in degree of FA desaturation across a total of 48 mammal species from terrestrial, semi-aquatic and fully-aquatic environments. Thermal conductivity, the rate at which heat moves through a material, is approximately 25 times greater in water than in air (Schmidt-Nielsen, 1975). Endotherms inhabiting aquatic environments therefore need a better insulating system to reduce body heat loss. I hypothesize that aquatic mammals will display higher degree of desaturation than

terrestrial species as they use blubber as their primary insulator and live in a more thermally challenging environment.

I also investigated pattern in FA desaturation across mammals inhabiting different latitudinal regions. The direct effect of ambient temperature is difficult to assess, since there is variation with season, water depth, and additionally semi-aquatic mammals use two environments where, according to their lifestyle, they can spend substantially more time in one than another. Therefore, in this study, latitude was used as a coarse proxy for ambient temperature. I hypothesize that species inhabiting extremely cold climates, such as the Arctic and the Antarctic, have the highest degrees of FA desaturation, while those species living close to tropical latitudes have low desaturation levels.

As the efficiency of an insulating system depends on a combination of different mechanisms, I also investigated whether other physical feature, the presence of fur, affected the type of FA contained in the adipose tissue. A dense and thick fur is indeed an important thermal insulator. Fur-covered animals have skin temperatures closer to their body core, but hairless animals have skin temperatures closer to that of the surrounding environment (Hokkanen, 1990). On land, terrestrial mammals rely mostly on their fur to reduce heat loss (Reidenberg, 2007), whereas in water, fully aquatic mammals have lost fur in favour of a thicker blubber layer (Reidenberg, 2007). Most semi-aquatic mammals rely on a combination of both fur and blubber (Liwanag et al., 2012b); therefore, here I investigate whether FA desaturation is related to hair density in semi-aquatic mammals. I hypothesize that as hair density increases, the degree of desaturation of adipose tissues decreases.

4.4 Materials and methods

4.4.1 Fatty acid data

This analysis included 48 mammalian species. FA composition data from 43 species were obtained by literature search using the search terms 'fatty acids', 'lipids', 'adipose tissue', and 'blubber'; on ScienceDirect and Google Scholar. Studies that did not provide all main FA values were excluded from analysis. Literature values were obtained mainly from biopsy samples collected from the mid-dorsal part of the body. In the case where the adipose tissue was divided into outer (section closer to the skin) and inner (section closer to the muscle) layers, only the FA composition of the outer layer was used for this analysis, as this section is thought to be less influenced by diet (Grahl-Nielsen et al., 2011; Guerrero et al., 2016; Koopman, 2007). Otherwise the whole core sample was used.

When male and female FAs were analysed separately, only male data were used, as females may experience changes in FAs associated with pregnancy and lactation. A list of the data sources can be found in Appendix 3.

I analysed the FA composition of 5 out of the 48 species included in this study. These species included 3 pinnipeds (Subantarctic fur seal; New Zealand fur seal, *Arctocephalus forsteri*; Australian sea lion, *Neophoca cinerea*) and 2 cetaceans (Pygmy right whale, *Caperea marginata*; Risso's dolphin, *Grampus griseus*) that correspond to animals that had stranded in the area of Sydney, Australia, and were collected by NSW Fisheries officers between 1999 and 2011. I collected a 0.3g sample from the outer blubber layer and used Budge et al. (2006) method to extract total lipids and prepare FA methyl esters. This procedure and the gas chromatography analysis were performed as indicated in Chapter 2, section 3.4.2.

4.4.2 Fatty acid desaturation

To determine the degree of FA desaturation I calculated a desaturation index (Δ9-DI). This indicates to what extent potentially non-dietary MUFAs have been synthesized by modification of their corresponding SFAs (Liwanag et al., 2012b). PUFAs were not included in this analysis, as most are of dietary origin (Käkëla and Hyvärinen, 1996a); therefore differences in proportions of polyunsaturated FAs are likely to be a result of differences in diet. Using the percentage by mass per FA, a Δ9-DI was calculated according to the formula of Käkëla and Hyvärinen (1996a):

$$\Delta 9 - \mathbf{DI} = \frac{(\mathbf{wt\%\ 14{:}\ 1\omega 5} + \mathbf{wt\%\ 16{:}\ 1\omega 7} + \mathbf{wt\%\ 16{:}\ 1\omega 9} + \mathbf{wt\%\ 18{:}\ 1\omega 9} + \mathbf{wt\%\ 18{:}\ 1\omega 7})}{(\mathbf{wt\%\ 14{:}\ 0} + \mathbf{wt\%\ 16{:}\ 0} + \mathbf{wt\%\ 18{:}\ 0})}$$

where wt%, is the percentage by weight of the respective FA.

4.4.3 Explanatory variables

To measure the effect of e*nvironment,* I classified the animals into three categories: where terrestrial mammals (n=8) are those whose complete life cycle takes place on land only; semi-aquatic species (n=25) are those who rely on both land (or ice) and water for breeding and feeding; and fully aquatic species (n=15) are those who spend their entire lives in water.

Latitude values correspond to the locations indicated in each study as the sample (biopsies) collection site. When coordinates were not available, an approximate latitude value was assigned, based on the location name provided.

The effect of *hair density* on the desaturation of FAs was tested only on semi-aquatic mammals. Literature values of hair density for 22 species of semi-aquatic mammals were obtained from different published studies using the search terms ‘fur density’ and

'hair density' on ScienceDirect and Google Scholar. When primary and secondary hair densities were calculated separately, I used total hair density for analysis.

4.4.4 Data analysis

To test whether the Δ9-DI of mammals could be explained by latitude, by environment, or by a combination of these variables; I applied a model selection approach. The models tested were: (a) latitude model ($\beta_0+\beta_{latitude}$) and (b) environment model ($\beta_0+\beta_{environment}$), for which each variable would be the only predictor for the desaturation of FAs; (c) an environment and latitude additive model ($\beta_0+\beta_{environment}+\beta_{latitude}$), for which both latitude and environment occupied by species would explain FA desaturation; (d) an environment and latitude interaction model ($\beta_0+\beta_{environment}*\beta_{latitude}$) which includes an interaction term to test for differences in allometry related to the environment; and (e) a Null model (β_0), for which no predictor variable was included and differences in desaturation of FAs would be attributable to other factors, such as those associated with evolution.

This model selection approach was assessed via second-order Akaike's Information Criterion with a correction for sample size (AICc) for each model. The smallest AICc value indicates the model with the highest support because it represents a more parsimonious explanation of the response investigated (Garamszegi and Mundry, 2014), although any other model with an AICc value within two units of the first model (i.e.: $\Delta AICc < 2$) is considered a good candidate (Burnham and Anderson, 2002).

To calculate AICc values, I applied models as phylogenetic generalized least squares (PGLS) regressions. In order to perform this analysis, a phylogenetic tree, including 48 mammal species, was created based on the supertree of Fritz et al. (2009), in which

branch lengths are proportional to time since divergence. Tree manipulations and the PGLS analysis were performed using the 'phytools' (Revell, 2012) and 'caper' (Orme, 2013) packages, respectively, in R (Team, 2016).

Outputs from the PGLS analysis include a parameter lambda (λ), which indicates the phylogenetic dependence in the data. When PGLS λ is close to 1 it indicates that the best fit to the data is provided by a "Brownian Motion" model of trait evolution, whereas values close to 0 suggest that the data have no phylogenetic structure (Freckleton et al., 2002). The PGLS analysis also produces an r value, which indicates the phylogenetic effect size. For the model with the highest support, I extracted the 95% confidence intervals (CIs) of the slope values. Significance was deemed when the CIs did not overlap 0.

To further investigate the effect of latitude on the FA desaturation of semi-aquatic mammals, I separated those species with higher fur density (>300 hairs/mm^2; i.e.: American Beaver, *Castor Canadensis;* Eurasian beaver, *Castor fiber;* muskrat, *Ondatra zibethicus;* Eurasian otter, *Lutra lutra*; cape fur seal, *Arctocephalus pusillus*; subantarctic fur seal, *Arctocephalus tropicalis* ; and New Zealand fur seal) from those with sparser fur. Thus, another analysis, using PGLS, was carried out. To do this, I manipulated the phylogenetic tree to include the 25 species of semi-aquatic mammals.

The relationship between hair density and desaturation of FAs in semi-aquatic mammals was investigated using PGLS regression. To do this, the tree was modified again to include only 22 species of semi-aquatic mammals for which hair density data was available. Hair density values were log-transformed for this analysis. As in earlier chapters, values are presented as mean ± standard deviation (SD) unless otherwise noted.

4.5 Results

4.5.1 Model selection

The interaction model including environment and latitude was the model with the lowest ΔAICc and the highest effect size (Table 4.1) and therefore the model with the highest support to explain the variation in desaturation of FAs in mammals. The PGLS λ value, or phylogenetic signal, was 0.50. The model with the second highest support was the additive model including environment and latitude. Although the ΔAICc value was also 0, the effect size was lower than the first model.

The interaction model indicates that the effect of latitude on FA desaturation depends on the physical environment, and vice versa (the effect of environment depends on latitude). In contrast, the additive model indicates that the effect of latitude on FA desaturation does not depend on the environment, and vice versa. The latitude model was also a good model, with a ΔAICc value of 2, but with lower effect size than the previous two models. The other model tested (environment) was not considered likely driver of the desaturation of FAs as the ΔAICc value was >2 units.

Table 4.1. Explanatory models for the desaturation of fatty acids in mammals. Outputs have been derived from phylogenetic generalised least squares (PGLS) analysis. The model with the highest support is that with the lowest Δ AICc and highest effect size.

Model	Δ AICc	PGLS λ	Effect size (r)
$\beta_0 + \beta_{latitude} * \beta_{environment}$	0.0	0.50	0.54
$\beta_0 + \beta_{latitude} + \beta_{environment}$	0.0	0.48	0.45
$\beta_0 + \beta_{latitude}$	2.0	0.57	0.31
β_0	4.0	0.50	NA
$\beta_0 + \beta_{environment}$	5.0	0.43	0.29

Mammals inhabiting different environments displayed different values of FA desaturation (Fig. 4.1). Terrestrial mammals had the lowest Δ9-DI (1.57 ± 0.85) whereas semi-aquatic and fully-aquatic mammals displayed higher Δ9-DIs (3.09 ± 1.74 and 2.94 ± 1.15, respectively).

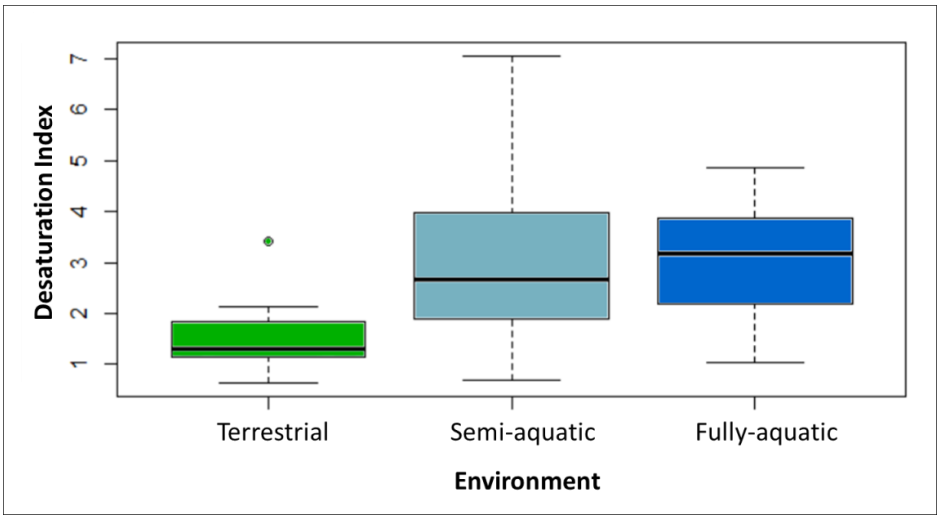


Figure 4.1. Comparison of desaturation index in the adipose tissues of terrestrial, semi-aquatic and fully-aquatic mammals. Boxes indicate the median (dark line), the lower and upper quartiles. Whiskers represent the maximum and minimum values and the only outlier is indicated by a circle. Terrestrial mammals have the lowest desaturation index. Fully- and semi-aquatic mammals have similarly high desaturation indexes, although semi-aquatic mammals display a wider range of values.

Although terrestrial mammals living in higher latitudes displayed slightly lower Δ9-DIs (slope = -0.02), these values were not significantly different from 0 (CIs = -0.10, 0.06), therefore indicating no relationship between latitude and Δ9-DI in the terrestrial environment (Fig. 4.2A). Similarly, although the Δ9-DI of fully-aquatic mammals (Fig. 4.2C) increased slightly with latitude (slope = 0.02), these values were not significantly different from 0 (CIs = -0.02, 0.05); therefore there was no correlation between latitude and Δ9-DI in fully-aquatic mammals. There was no significant difference in slopes

between fully-aquatic and terrestrial mammals (CIs = -0.11, 0.04), but they had significantly different intercept values (CIs = -5.05, -4.31). Semi-aquatic mammals showed a positive (slope = 0.06) and significant (CIs = 0.01, 0.11) relationship between latitude and Δ9-DI (Fig. 4.2B), which indicates that only this group exhibits higher degrees of FA desaturation when living in colder environments.

When sparsely- and densely-furred semi-aquatic mammals were analysed as two separate groups, the desaturation index of the sparsely-furred semi-aquatic mammals increased significantly (CIs = 0.01, 0.15) with latitude (Fig. 4.3) and the slope was more pronounced (0.08) than when they were analysed together with densely-furred semiaquatic mammals (0.06). PGLS λ value was 0. On the other hand, densely-furred semi-aquatic mammals did not display a correlation of desaturation index with latitude (CIs = -3.49, 3.41).

4.5.2 Effect of hair density on fatty acid desaturation in semiaquatic mammals

Desaturation index exhibited a significant (CIs = -0.123, -0.916) negative relationship (slope = -0.52) with hair density (Fig. 4.4), and the PGLS λ was 0. Semi-aquatic species with higher hair density had adipose tissues with less FA desaturation. The Atlantic walrus had the lowest hair density (1.8 hairs/mm^2) and one of the highest Δ9-DI after the arctic seals: ringed and harbour seals. Beavers, fur seals, muskrats, and otters were the animals with the densest fur and they display the lowest degrees of desaturation in their adipose tissues. The centre of the graph in Fig. 4.4 comprises mostly pinnipeds, the phocids or true seals, and the otariids sea lions, which shared similar hair densities, but displayed a wide range of Δ9-DI.

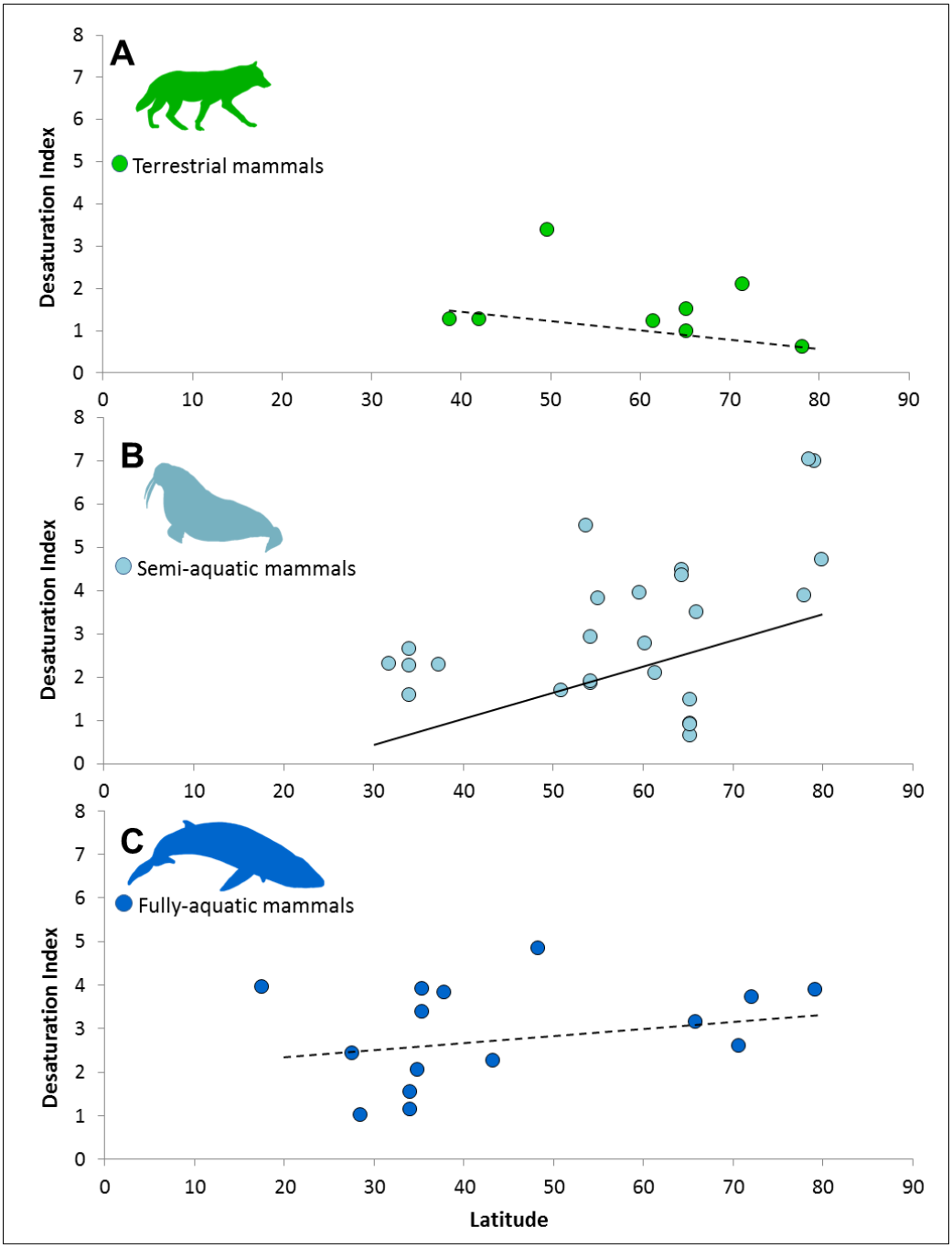


Figure 4.2. Desaturation index of FAs as a function of latitude for mammals inhabiting different environments. Dashed (non-significant correlation) and solid (significant correlation) lines indicate the phylogenetic generalised least squares (PGLS) regression lines for A) terrestrial ($y = -0.02(x) + 2.27$) B) semi-aquatic ($y = 0.06(x) - 1.32$) and C) fully-aquatic mammals ($y = 0.02(x) + 1.91$). Sample sizes were 8, 25 and 15 for terrestrial, semi-aquatic and fully-aquatic mammals, respectively. Only the blubber of semi-aquatic mammals displayed significantly (CIs = 0.01, 0.11) higher FA desaturation when living in colder latitudes.

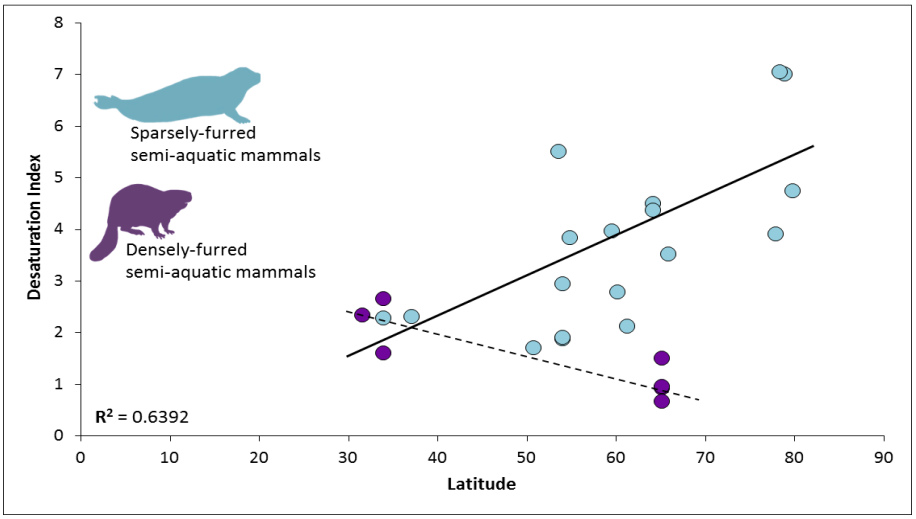


Figure 4.3. Desaturation index of semi-aquatic mammals as a function of latitude. Sparsely-furred species include true seals, sea lions, walruses and polar bears whereas densely-furred species include otters, beavers, muskrats and fur seals (hair density > 300 hairs/mm^2). Dashed (non-significant correlation) and solid (significant correlation) lines indicate the phylogenetic generalised least squares (PGLS) regression lines for sparsely- ($y = 0.08(x) - 0.93$) and densely-furred semi-aquatic mammals ($y = -0.04(x) + 3.36$). Only sparsely-furred semi-aquatic mammals displayed a significant (CIs = 0.01, 0.15) positive correlation between desaturation of fatty acids and latitude.

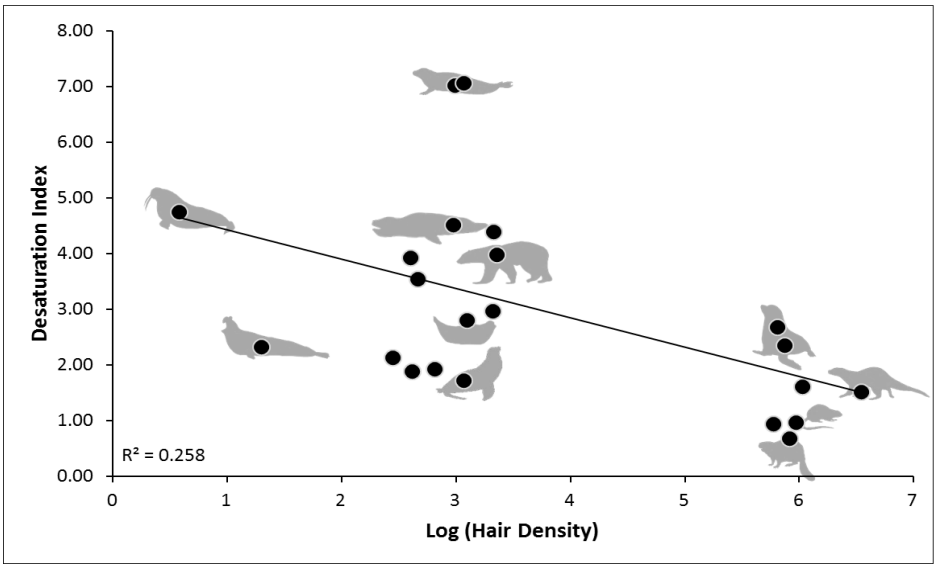


Figure 4.4. Desaturation index as a function of hair density in semi-aquatic mammals (n=22). Desaturation of FAs exhibited a negative relationship with hair density ($y = -0.52\log(x) + 4.86$). Silhouettes correspond to the species represented by the data points.

4.6 Discussion

4.6.1 Fully-aquatic insulation: reliance on blubber

Fully-aquatic mammals inhabit the environment with the highest cooling potential where, in the absence of fur, they have to rely solely on their blubber as a thermal insulator (Liwanag et al., 2012b). In bare-skinned animals, the skin and superficial tissues become very cold when they are exposed to low environmental temperatures (Irving and Hart, 1957). In fact, the temperature of whales' skin has been found to equal that of the water (Hokkanen, 1990). The capacity to keep cold but pliable tissues is; therefore, very important and is strongly related to high levels of FA desaturation. Fully-aquatic mammals displayed a higher degree of desaturation in their blubber layer than terrestrial mammals (Fig. 4.1), which indicates the importance of FAs in maintaining the outer blubber layer at cold temperatures.

For body temperature to remain constant, heat loss must equal heat production (Schmidt-Nielsen, 1975), and this is particularly challenging for animals living in a medium with great cooling power such as water. The role of blubber as the only thermal barrier is hence critical. Nevertheless, a difference in the capacity of blubber to lower its solidification point when fully-aquatic mammals live in colder environments was not evident in this study. Although there was a small positive correlation between desaturation and latitude, this was not significant (Fig. 4.2C). The desaturation of FAs, although is greater than terrestrial mammals, seems to reach similar levels regardless of the environmental temperatures (latitudes).

Latitude in some contexts can be used as a proxy for ambient temperatures, where temperature can vary widely from tropics to the poles; however, deep-water temperatures do not experience great variations across latitudes (Labeyrie et al., 1987). Fully-aquatic mammals are constantly exposed to cold deep-water temperatures and

varying surface temperatures according to their habitat. This could explain the minimal modification in FA desaturation found in this group. A whale living in tropical waters must still be able to withstand cold surrounding temperatures with each dive. Nevertheless, this scenario should not be very different than that of semi-aquatic mammals, which are also good divers and inhabit tropical and polar waters, but they do modify their FAs with latitude.

Air temperature variations could explain why semi-aquatic mammals are significantly influenced by latitude and fully-aquatic mammals are not. For example, for Antarctic pinnipeds living in the Southern Ocean, the coldest marine environment on Earth, seals move daily between water temperatures as low as -2°C (Clarke et al., 2009) and air temperatures as low as -25°C in winter (Bargagli, 2006). Fully-aquatic mammals therefore, are not experiencing the most extreme temperature variations and therefore a modification of FA desaturation with latitude, although exists, is minimum compared to semi-aquatic mammals.

There is another kind of FA metabolism that could impact the thermal properties of blubber and which was not taken into consideration in this study. Certain families of cetaceans synthesise considerable levels of isovaleric acid, a short branched-chain FA . This FA has been found in great quantities (e.g.: ~35% in Hector's dolphin, *Cephalorhynchus hectori*) in the outer blubber layer of some odontocetes (Koopman et al., 2003). They are hypothesised to play a role in maintaining blubber pliability in cold environments (Budge et al., 2006; Koopman et al., 2003). Therefore, potentially some fully-aquatic mammals would rather synthesise this particular branched-chain FA than insert a desaturation into their SFAs.

As has been stated earlier, the efficiency of blubber as a thermal insulator depends on a variety of parameters, including thickness, water and lipid content, and FA composition

(Liwanag et al., 2012). The FA desaturation of the blubber of fully-aquatic mammals changed minimally with latitude, but due to their lifestyle, thickness of blubber can experience the greatest modifications. When fasting, during reproduction and lactation, several large baleen whales rely heavily on the energy stored in their blubber (Koopman et al., 2002), which implies a significant reduction of their blubber thickness. This change, although caused by food deprivation and not thermoregulation, has a positive influence upon the thermal needs of whales while in warm regions. According to the models of heat loss in marine mammals provided by Watts et al. (1993), a small change in blubber depth can cause substantial variation on the lower critical temperature (below which an endotherm must increase its metabolic rate to maintain its body temperature). Thus, the depth of the insulating layer strongly affects thermoregulation. In fact, small cetaceans have been found to adapt to ambient temperatures by varying the thickness of their blubber (Williams and Friedl, 1990). While a thinner layer allows greater dissipation of heat in warmer waters, a thick blubber layer provides the necessary insulation in colder regions.

Lipid content is another parameter that can change the overall insulation provided by blubber. Worthy and Edwards (1990) found that harbor porpoises inhabiting temperate regions had significantly higher lipid content and greater blubber thickness compared to spotted dolphins from tropical waters. Therefore, the insulation of blubber depends on several parameters. In this case FA desaturation did not change significantly when fully-aquatic mammals live in colder waters, but they may be modifying other parameters, such as thickness, synthesis of branched-chain FAs, lipid content and/or water content in order to achieve a more effective insulation.

4.6.2 Blubber or fur: the dilemma of semi-aquatic mammals

Switching from one environment to another is a challenging task, as this implies that animals are exposed to contrasting temperatures and very different heat loss rates. Thermoregulatory demands, therefore, can fluctuate frequently. Their insulating layers, blubber and fur, need to be particularly efficient in both environments.

Semi-aquatic mammals display a higher degree of desaturation than terrestrial mammals (Fig. 4.1), as their aquatic lifestyle requires efficient thermal properties because their heat is drawn from the body faster in water than in air (Nienaber et al., 2010).

I have demonstrated that when animals live in colder environments they have blubber layers that can withstand lower temperatures without solidifying, which is evidenced by the positive relationship found between desaturation of FAs and latitude (Fig. 4.2B). But most semi-aquatic mammals rely not only on blubber to retain body heat, but also on fur.

Possessing both fur and blubber insulation is an interesting aspect of mammalian thermoregulation. Fur can be an effective insulator for terrestrial lifestyle, but is it also in water? The presence of dense, waterproof fur is a characteristic of recent invaders of the aquatic environment, such as otters, polar bears, beavers and other rodents (Liwanag et al., 2012b; Reynolds, 1993). In this study, muskrats, beavers and otters were the species with the lowest degrees of desaturation and the highest hair densities (Fig. 4.3 and 4.4) which supports the idea that these mammals rely on dense waterproof fur, rather than the adipose tissue, as an insulator (Reynolds, 1993). The efficiency of this coat relies on the air trapped in its hairs (Fish et al., 2002), which forms a protective warm layer and keeps the skin relatively dry (Dawson and Fanning, 1981). This thermal barrier, however, can be a disadvantage in swimming performance.

Recent entries to the aquatic environment possess drag-based propulsion in water and they have less fusiform body shapes, are shallower divers and slower swimmers compared to earlier entries, such as seals (Reynolds, 1993). However, the transition from a terrestrial to an aquatic lifestyle required adaptations for enhanced locomotory performance in water, which includes greater streamlining. Longer hairs and denser pelage implies higher surface area, and hence, higher frictional drag, which is not convenient when swimming.

Earlier entries to the aquatic environment, such as pinnipeds, have become much better swimmers, and this has implied the reduction of drag through more streamlined bodies (Fish, 1994), reduction of fur density and thickness (Liwanag et al., 2012a), and the transition to blubber as an insulator (Liwanag et al., 2012b). Although fur seals still maintain a dense water-repellent underfur covered by wettable guard hairs, the insulation provided by their fur is not comparable to that offered by the thick pelage of land mammals (Irving et al., 1962; Liwanag et al., 2012a). Sea lions and true seals rely more heavily on blubber to prevent excessive heat escape (Rosen and Trites, 2014). They possess a wettable fur that is not a good insulator in water, as the air layer trapped in the fur is released due to compression when diving (Liwanag et al., 2012b); thus, blubber becomes more important as a thermal barrier. This was supported by the significant effect of latitude on FA desaturation in sparsely-furred semi-aquatic mammals (Fig. 4.3).

A thick blubber layer could ensure an effective insulation in water, which is the case of most fully-aquatic mammals, but for a semi-aquatic mammal an extremely thick blubber layer can be a problem. Most semi-aquatic mammals are relatively small compared to cetaceans, and an extremely large blubber layer would impede an agile terrestrial locomotion, especially for those animals living in rookeries with steep slopes,

which is common amongst sea lions and fur seals. A trade-off, having a not too thick, but efficient thermal insulator is therefore imperative. A variation in FA composition can be an effective mechanism to maintain a good insulating layer without limiting the manoeuvrability of these animals. Higher FA desaturation in higher latitudes allows the blubber layer near the skin to lower its temperature and therefore reduce heat loss to the surrounding environment. This particular feature can be more important in semi-aquatic mammals than in any of the other two groups, as they cannot afford a very thick pelage like land mammals, and neither can they develop extremely thick blubber as fully-aquatic mammals.

4.6.3 Adipose tissue in terrestrial mammals: not an ideal thermal insulator

Terrestrial mammals displayed the lowest degree of desaturation of all groups (Fig. 4.1) and they did not exhibit a relationship between FA desaturation and latitude (Fig. 4.2A). Although terrestrial mammals living in arctic climates encounter great diurnal and seasonal temperature variations (Irving et al., 1962), they seem to have little need for lowering the solidifying point of their adipose tissue. This study included several arctic terrestrial mammals, well adapted to polar conditions; for them body fat did not appear to have an important role in keeping warm. Our observations concur with those of Scholander et al. (1950b), who state that body fat does not seem to play any role in the insulation of terrestrial arctic mammals.

The Arctic fox, *Alopex lagopus,* is covered by a coat made up of dense underhair and long cover hair (Cholewa and Popescu, 2014). This pelage is effective in protecting against cold. In fact, seasonal moulting has been reported in some canids (foxes and the raccoon dog), which provides them with a thinner pelage for summer and a thicker coat for winter (Xiao, 2009). Irving and Krog (1955) measured the skin temperature on the

body core of arctic dogs and found that it was always around 30°C, with an environmental temperature of -40°C. This temperature gradient highlights the crucial role of fur in retaining body heat in terrestrial mammals. In fact, fur has been found to be a more efficient insulator than adipose tissue (Scholander et al., 1950b), since it creates the same thermal gradient between body core and body surface, but across a much smaller thickness (Liwanag et al., 2012b).

In the case of animals with poor insulating fur (thin, sparse fur or hairless), the thermal role of adipose tissues is expected to gain importance. Henriques and Hansen (1901) compared two groups of pigs living at 0°C: one group with their skin directly exposed to the cold ambient air and the other where animals had a sheepskin garment. Bare-skinned pigs had adipose tissues that solidified at a temperature 2.4 °C lower than pigs protected by a coat. This indicates an increase in the degree of desaturation in the FAs of pigs whose skin was in direct contact with the cold environment. Low tissue temperatures, therefore, seem to be the trigger for the desaturation of FAs. Conversely, when the skin temperature is maintained close to the warm body temperature, as it is the case of garment-covered pigs and fur-covered mammals, a modification of FAs may not be necessary.

Examples of low skin temperatures as the cause of an increase in desaturation are the thinly insulated extremities of polar mammals. The feet and noses of arctic dogs, foxes, and reindeer may reach temperatures very similar to the freezing environment surrounding them (Irving and Krog, 1955). In order to avoid the rigidity or solidification of their extremities in these adverse conditions, the adipose tissues of these animals are expected to have a lower solidifying point, which is achieved by an increased FA desaturation. Irving et al. (1957) found that the poorly insulated footpads of animals living in cold environments had the lowest melting (or solidifying) points compared to

other body sites. Therefore, the temperature of the skin, and hence of the subcutaneous adipose tissue, is crucial for the generation of MUFAs. This explains why terrestrial mammals, most of them protected by a thick fur and therefore with skin temperatures close to their body core, do not increase the desaturation levels of their body core when living in high latitudes.

The outlier represented in Figure 4.1 corresponds to a 13-lined ground squirrel, the only terrestrial hibernator included in this study, which had the highest FA desaturation value among land mammals. Squirrels can lower their body temperature as low as 4°C during torpor (Price et al., 2013; Frank, 2002); therefore, a high FA desaturation is likely to be a mechanism to avoid solidification of their tissues. Besides, fur of ground squirrels has been found to provide very little insulation compared to mammals of similar body size (Scholander et al., 1950). Therefore, this high level of FA desaturation is not unexpected, although the impact of poor fur quality is probably minimal compared to the impact of low tissue temperatures.

4.7 Conclusions

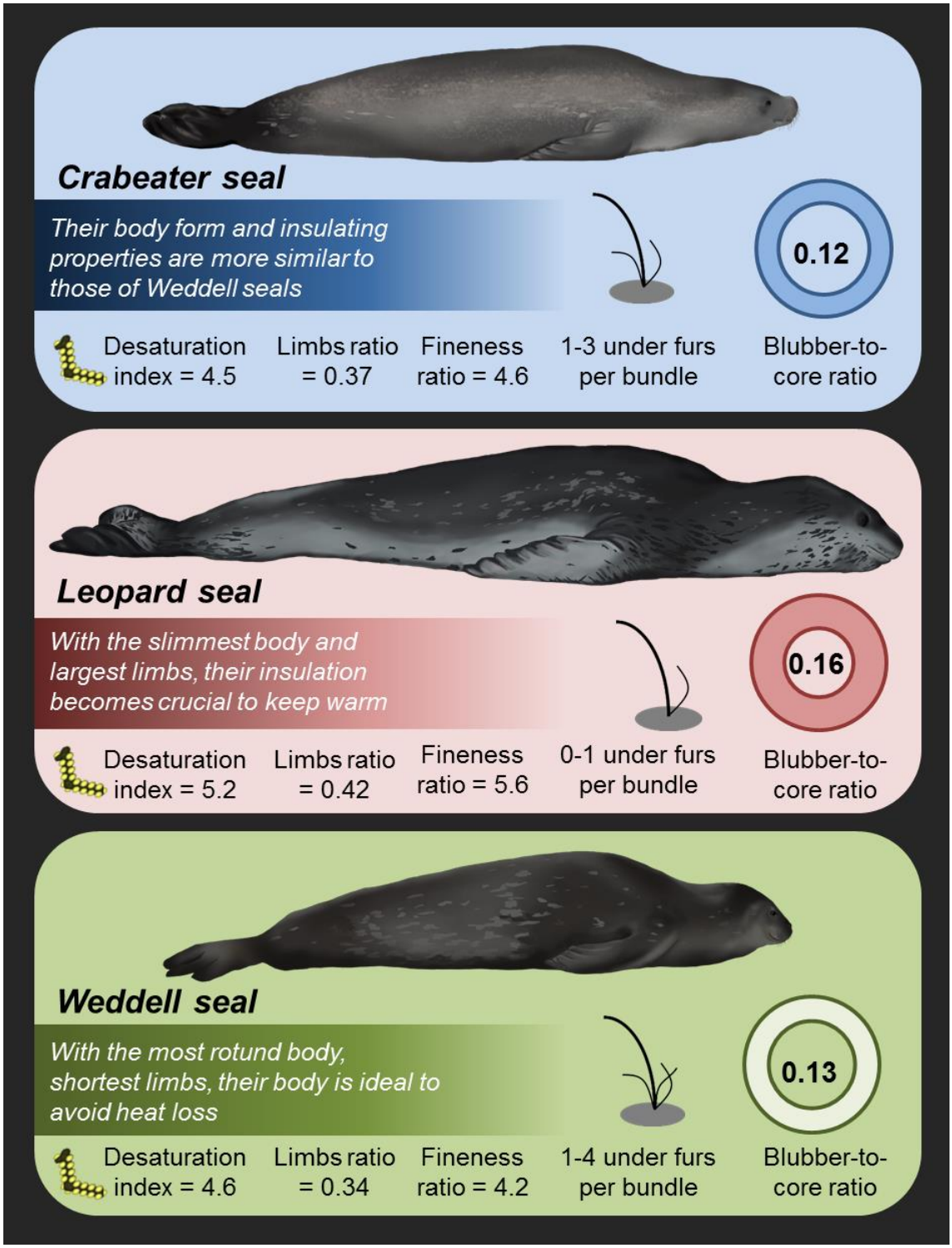
I show that the ability to modify FAs is an important part of mammalian thermal plasticity, especially for mammals living under adverse thermal conditions. I demonstrate that fully-aquatic mammals have increased FA desaturation in their blubber compared to terrestrial mammals, but they do not modify their FAs in different latitudes; they are potentially regulating other parameters of blubber instead. Semi-aquatic mammals rely on both fur and blubber as thermal barriers; these two insulators are complementary: when they have denser fur their FA desaturation is lower, and vice versa. These mammals increase their FA desaturation when living in colder regions, so that they can cool their superficial tissues near freezing temperatures without

solidification. The ability to modify their FAs is imperative in semi-aquatic species, as they do not have extremely thick blubber layers as fully-aquatic mammals or a very abundant fur as terrestrial mammals. Terrestrial mammals rely mainly on fur as insulator, so their adipose tissues have low levels of FA desaturation, which does not vary in colder latitudes.

CHAPTER 5

Blubber, fur and body form: thermal strategies in Antarctic pack-ice seals

5.1 Graphical abstract



5.2 Abstract

Polar pinnipeds are unique since they have to cope not only with the extreme cold, but also with the great thermal fluctuations that moving constantly from ice to water entails. I aimed to determine if there are relationships between FA desaturation, blubber-to-core ratio, fur and body form in the thermoregulation strategies of three sympatric Antarctic seals, the leopard, crabeater and Weddell seals. To minimize the impact of environmental factors, seals were sampled at the same location and at the same time of year. Skin and blubber samples were analysed for FAs, blubber-to-core ratio and hair density. Morphometrics were obtained for body mass, fineness and limb ratios. There were inter-specific differences. Leopard seals had different morphometric and insulating patterns from the two other species, including the slimmest body, the longest limbs and the lowest number of under furs. A long, slim body with long forelimbs are likely an advantage for speed and manoeuvrability for a fast-pursuit predator like the leopard seal however these features could propose thermal limitations when living in a cold environment. The leopard seal appears to compensate as they have the greater blubber-to-core ratio in their outer blubber. This suggests that leopard seals rely more heavily on blubber to keep warm than the crabeater and Weddell seals. There were interesting intra-species patterns. Body length correlated with the variation of fur and blubber parameters suggesting potentially age-related changes. Body length was positively correlated with FA desaturation and negatively correlated with blubber-to-core ratio and, in crabeater seals, with hair density. Therefore the larger, presumably older seals have thinner blubber-to-core ratio but higher FA desaturation; these observations are consistent with allowing the blubber to tolerate lower temperatures without solidifying and to reduce heat loss more efficiently. These results suggest that older seals rely more on blubber and less on fur as an insulator than the smaller, younger seals.

5.3 Introduction

The challenge of a semi-aquatic lifestyle is that an animal must adapt to fundamentally different thermal demands when moving from the terrestrial to the aquatic environment and vice versa. For example, the surrounding ambient temperature of a seal changes significantly with just a plunge from the land's surface into the water, but as homoeothermic animals, their internal temperature must be kept constant. As has been discussed previously, aquatic and terrestrial environments do not only differ in temperature, but also in the capacity to conduct heat away from the body. Therefore animals in water will lose heat faster than on the land's surface. Semi-aquatic mammals have to make continuous adjustments to live in these two vastly different environments; this remarkable plasticity makes them a particularly interesting group of study.

In order to maintain a constant deep body temperature, the overall metabolic heat production of an animal must equal heat loss (Scholander et al., 1950b). One of the adaptations that marine mammals have developed to reduce body heat loss is a large-sized body (Innes et al., 1990). As animals become larger their volume increases more rapidly than their surface; reducing their surface-to-volume ratios (Bergmann's rule: Bergmann, 1847). This is advantageous to marine mammals because it decreases the relative area across which heat is transferred to the aquatic environment (Pabst et al., 1999). In addition, marine mammals have developed a streamlined and compact body form with few protruding appendages from which heat can dissipate rapidly (Allen's rule: Allen, 1877; Riedman, 1990).

Marine mammals possess increased insulation through blubber or dense fur (Ryg et al., 1988; Scholander et al., 1950b). Interestingly, pinnipeds possess both. The fur of seals consists of a longer stiffer and flattened guard hair which grows anterior to a group of shorter finer under furs (King, 1983). This combination of hairs keeps the skin relatively

dry (Dawson and Fanning, 1981). However, in the aquatic environment, the air trapped in fur is easily compressed and hence released when animals dive (Liwanag et al., 2012b), which reduces the effectiveness of this insulator in water.

Blubber was a critical component of the mammalian transition from the terrestrial to the aquatic environment (Koopman et al., 2002), since it has important insulating properties and also plays a role in streamlining (Arriola et al., 2013; Ryg et al., 1988). Blubber provides an envelope of fixed insulation that is not subject to compression, as with air trapped in fur (Elsner, 1999; Liwanag et al., 2012b).

Blubber thickness varies between and within species and over the lifetime of an animal (Castellini et al., 2009; Dunkin et al., 2005; Pearson et al., 2014), which can impact the amount of heat that is transferred to the environment. The biochemical composition of blubber is also an important factor in the thermoregulation of pinnipeds. An increase in desaturation of the FAs comprising the blubber has frequently been seen as an adaptive reaction to the cold (Best et al., 2003; Guerrero et al., 2016; Käkëla and Hyvärinen, 1996b). A higher desaturation index indicates a greater number of endogenous monounsaturated FAs (MUFAs) relative to their saturated (SFA) counterparts (Käkëla and Hyvärinen, 1996a). Higher FA desaturation improves fluidity of tissues so that they can resist lower temperatures without solidifying (Irving et al., 1957). The capacity of superficial tissues to withstand cold temperatures is important in heat loss reduction, as this creates a thermal gradient from the cold outer blubber layer to the warm body core. This thermal gradient facilitates peripheral vasoconstriction, thus reducing the circulation of warm blood near the surface, which impedes the transfer of body heat to the environment (Elsner, 1999).

My study species are three members of the Antarctic true seals in the tribe Lobodontini, the crabeater, leopard and Weddell seals. They are observed from the coastal Antarctic

waters, throughout the pack ice of the Southern Ocean. The leopard seal however is also observed further north in the warmer mid-latitudes on occasions (Gray et al., 2005). Although inhabiting similar environments, these Antarctic pack-ice seals have differences in physiology and lifestyles.

The Weddell (maximum diving depth = ~600 m; Kooyman, 1981) and crabeater seals are deep divers (typically ~100 m, although they can dive up to ~600 m; Bengtson and Stewart, 1992) compared to the leopard seal, which is a very modest diver for a marine mammal, as they perform short (~2 min), shallow (30 m or less) dives (Krause et al., 2015). Compared to the two other species, the leopard seal has a lower whole body oxygen carrying capacity because they have a smaller spleen, lower haemoglobin concentration, and lower packed cell volume (Gray et al., 2006). Where the crabeater seals are planktivores, predominantly krill-feeding specialists (Hückstädt et al., 2012), the Weddell and leopard seals feed on an array of prey, including fish, cephalopods, and krill (Burns et al., 1998; Casaux et al., 2009; Davis et al., 1999; Hall-Aspland and Rogers, 2007; Lake et al., 2003). The leopard seal also takes warm-blooded prey, more so than any other pinniped (Rogers, 2009), including crabeater, Weddell and southern elephant seals (Hall-Aspland and Rogers, 2007), fur seals (Krause et al., 2015) and penguins (Rogers and Bryden, 1995). They have been observed to use high-energy pursuit behaviour to capture these types of prey (Rogers and Bryden, 1995). These differences in physiology and lifestyles between the species may have implications for their thermal requirements.

First, I aim to determine if there are differences in thermal strategies between the leopard, crabeater and Weddell seals, which I assess through the analysis of their blubber, fur and morphometric parameters. Second, I aim to identify if there are intra-species patterns in the thermoregulation of these Antarctic seals. I predict that the

species with slender body form, potentially the leopard seal, will be more susceptible to heat loss due to its potentially higher surface-to-volume ratio, and therefore will possess an enhanced insulating layer, either in the form of an improved blubber, or higher fur quality. Furthermore, I hypothesise that, as seen in Chapter 4, fur and blubber parameters play a complementary role, with seals with sparser fur having an improved blubber layer and vice versa. Thus, this study intends to understand how physical and biochemical parameters contribute to the thermoregulation of Antarctic seals inhabiting the same temporal and spatial environment, and whether these species utilise their thermal adaptations differently.

5.4 Materials and methods

5.4.1 Sample collection

In total, 21 crabeater seals (11 females and 10 males), 13 leopard seals (2 females and 11 males), and 20 Weddell seals (13 females and 7 males) were sampled off the Danco Coast, Western Antarctic Peninsula (64°09′ S 60°57′ W) during the austral summer (January and February) of 2015. Age ranges were classified as adults and sub-adults. Seals were immobilised as described in Chapter 2, section 2.4.1. Following immobilisation, sex, standard length (straight line nose to tail, Fig. 5.1) and maximum girth were recorded and 8 mm diameter biopsies containing whole cores of blubber and skin were collected from the mid-dorsal surface.

Biopsy samples were measured with a caliper and then separated into skin and fur, outer and inner blubber layers. Each sample was stored in airtight vials and frozen at -20°C for further analysis.

5.4.2 Fineness ratio

Body shape was assessed by calculating a fineness ratio (Castellini and Kooyman, 1990), which formula was described in Chapter 2, section 2.4.3. This ratio provides an estimation of how streamlined the overall shape of the body is. Higher fineness ratios indicate slimmer seals.

5.4.3 Limb ratio

To examine the proportion of limb protrusion relative to the body length I measured the length of hind and fore flippers (Fig. 5.1) and calculated the proportion of the length of appendages relative to the body, referred to here as the limb ratio (LR):

$$LR = \frac{FF + HF}{SL}$$

where FF is the length of fore flippers, HF the length of hind flippers and SL is standard length.

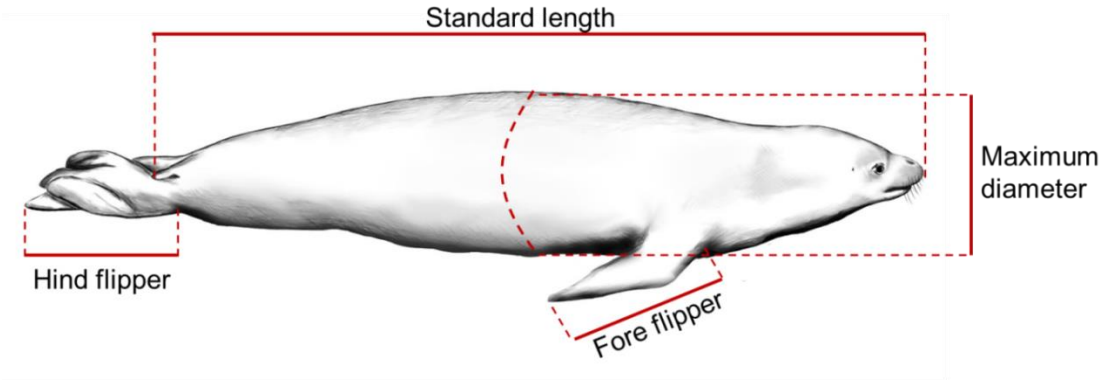


Figure 5.1. Body measurements for all three species of Antarctic pack-ice seals. Maximum diameter and standard length were used to calculate a fineness ratio. Fore and hind flipper lengths were used to calculate limb ratio, which is a ratio of appendages length relative to standard body length.

5.4.4 Blubber depth ratio

To examine variations in blubber thickness in relation to the metabolically active body core (Fig. 5.2), I calculated a blubber-to-body-core ratio (BCR) according to the formula of Castellini et al. (2009):

$$BCR = \frac{2 * BD}{D - (2 * BD)}$$

Where BD is blubber depth and D is maximum diameter.

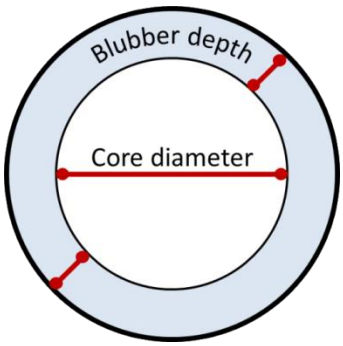


Figure 5.2. Diagrammatic view of the transverse plane of a seal body from Castellini et al. (2009). Blubber depth and core diameter were used to calculate a blubber-to-core ratio, which examines how the blubber ring that insulates the animal changes in relation to the metabolically active body core.

5.4.5 Fatty acid analysis

Only outer blubber layers were utilised for FA analysis, as this section of blubber is thought to be less influenced by diet (Grahl-Nielsen et al., 2011; Guerrero et al., 2016; Koopman, 2007). Total lipid extraction, fatty acid methyl esterification and gas chromatography analyses were conducted as described in Chapter 3, section 3.4.2

5.4.6 Desaturation index

The FA desaturation index of the outer blubber layer was calculated as described in Chapter 4, section 4.4.2.

5.4.7 Fur density

Skin samples were all collected using 8mm biopsy punches; therefore they were equal in size with an area of 50 mm^2. Since the black skin of seals complicates the counting of hairs under the microscope, samples were shaved until the dark skin was removed and the follicles were clearly visible. Once shaved, skin samples were fixed in 10% formalin for a minimum of 5 days before analysis.

Mammalian fur is organised into bundles, where every guard hair is accompanied by a number of under furs. To count the number of bundles I took digital photographs of each skin sample under a dissecting microscope using 1X objective. The bundles were then marked and counted using ImageJ software. To count under furs I used a compound microscope (Leica CTR5000) with a 10X objective. I selected three bundles per sample and counted the number of under furs per guard hair unit.

5.4.8 Analysis

Due to the multiple variables tested, I applied a principal component analysis (PCA) to identify the main differences between the three seal species. In this manner the 7 variables (body mass, fineness ratio, limb ratio, blubber-to-core ratio, guard hair density, number of under furs, and desaturation index) could be described in two dimensions. In order to normalise the skewed data, variables were log-transformed prior to the analysis. Multivariate analysis of variance (MANOVA) was then carried out on

the PC scores with species as factor. In addition, I used ANOVA, followed by Tukey's post hoc analysis, to test the differences in each of the variables between species.

In order to identify intra-species thermal patterns, I applied a PCA to each species, using the same 7 variables, followed for a MANOVA applied to the PC scores to test differences between age ranges and gender, within each species.

In order to determine how some parameters related to insulation change as seals grow, I carried out simple linear regressions to evaluate the effect of standard body length on guard hair density, blubber-to-core ratio and desaturation index.

All statistical tests have α level of statistical significance of 0.05. When the 95% confidence intervals (CIs) were extracted from slope values, significance was deemed when the CIs did not overlap 0. As in earlier chapters variance is standard deviation (SD) unless specified. All data analyses were conducted in R (Team, 2016).

5.5 Results

5.5.1 Inter-specific differences

Principal Component Analysis demonstrated clear differences between the three Antarctic seal species. The first two components accounted for 68% of the variation (PC1 43%, PC 25%) of physical parameters with potential impact on the thermoregulation. The bivariate plot (Fig. 5.3) shows leopard, crabeater and Weddell seals as separated groups. The segregation of the PC1 was driven by the positive Eigenvalues of fineness ratio, limb ratio, guard hair density and blubber-to-core ratio and the negative Eigenvalue of number of under furs. Positive Eigenvalues of body mass and desaturation index were the main drivers of the segregation of the PC2. A

MANOVA carried out on the first two PC scores confirmed significant differences between the three seal species (*Wilks' λ* = 0.097, $P < 0.01$).

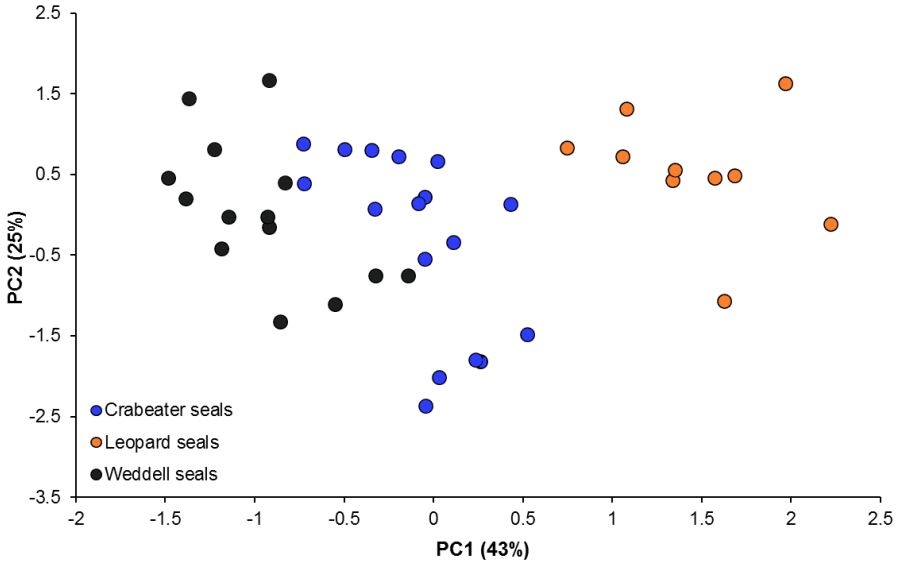


Figure 5.3. Physical differences between Antarctic seals. A) Principal component plot for blubber, fur and morphometric variables of crabeater, leopard and Weddell seals. The first 2 principal components combined explained 68% of the variance. Leopard seals are separated from crabeater and Weddell seals due mostly to higher values of fineness and limb ratios, and guard hair density.

5.5.2 Blubber parameters

Blubber-to-core ratio (Table 5.1) was significantly (F_2 = 4.23, P = 0.020) higher in leopard seals (0.16 ± 0.06) compared to crabeater (0.12 ± 0.04) and Weddell seals (0.13 ± 0.04). These values indicate the proportion of blubber relative to their active body core. Leopard seals had a blubber layer that corresponded to 16% of their body core while the blubber layer of crabeater and Weddell seals only corresponded to 12% and 13% of their body core, respectively.

The blubber layer of leopard seals had higher (5.16 ± 0.83) FA desaturation index than crabeater (4.54 ± 0.93) and Weddell seals (4.59 ± 0.94), but the difference was not significant (F_2 = 2.15, P = 0.128).

5.5.3 Fur parameters

Antarctic seal species had significantly different guar hair densities (F_2 = 28.49, P < 0.001). Leopard seals had significantly higher guard hair density, followed by crabeater and Weddell seals, respectively (Table 5.1). The opposite pattern was found in under furs. There was a significant difference in the number of under furs per bundle between species (F_2 = 32.16, P < 0.001). Weddell seals had between 1 and 4 under furs per bundle, with an average of 2.33 ± 0.68. Crabeater seals had between 0 and 3 under furs per bundle and an average of 1.43 ± 0.54. Leopard seals only had between 0 and 1 under fur per bundle and an average of 0.64 ± 0.31.

5.5.4 Body measurements

Body mass (Table 1) was significantly higher (F_2 = 13.16, P < 0.001) in leopard seals (317.48 ± 53.06 kg) compared to Weddell (260.24 ± 48.76 kg) and crabeater (231.02 ± 42.20 kg) seals.

Limb ratio was significantly greater (F_2 = 33.74, P < 0.001) in leopard seals, where the proportion of hind and fore flippers length, relative to the body standard length, was equal to 0.42 ± 0.03, followed by crabeater seals with a limb ratio of 0.37 ± 0.03 and Weddell seals with the shortest appendages, which correspond to 0.34 ± 0.03.

The fineness ratio of leopard seals was significantly (F_2 = 115.7, P < 0.001) higher (5.56 ± 0.35) than crabeater (4.58 ± 0.25) and Weddell seals (4.17 ± 0.20). According to

this ratio, Weddell seals were the most rotund while leopard seals were the slimmest seals.

Table 5.1: Blubber, fur and body measurements of crabeater (n=21), leopard (n=13) and Weddell seals (n=20). Values are presented as mean ± standard deviation.

	Crabeater seals	Leopard seals	Weddell seals
Blubber parameters:			
Desaturation index	4.54 ± 0.93 [a]	5.17 ± 0.83 [a]	4.59 ± 0.94 [a]
Blubber-to-core ratio	0.12 ± 0.04 [a]	0.16 ± 0.06 [b]	0.13 ± 0.04 [a]
Fur parameters:			
Guard hair density (hair*cm^2)	288.7 ± 51.25 [a]	345.26 ± 54.82 [b]	211.24 ± 35.76 [c]
Under furs per bundle	1.43 ± 0.54 [a]	0.64 ± 0.31 [b]	2.33 ± 0.68 [c]
Body measurements:			
Limbs ratio	0.37 ± 0.03 [a]	0.42 ± 0.03 [b]	0.34 ± 0.03 [c]
Fineness ratio	4.58 ± 0.25 [a]	5.56 ± 0.35 [b]	4.17 ± 0.2 [c]
Body mass (Kg)	231.02 ± 42.2 [a]	317.48 ± 53.06 [b]	260.24 ± 48.76 [a]

[a b c] Different superscript letters indicate significant differences between species ($P<0.05$, Tukey's post hoc analysis).

5.5.5 Intra-species insulation patterns: crabeater seals

The MANOVA performed on the PC scores showed that crabeater seals of different age groups (adults and sub-adults) had significantly different (*Wilks'* $\lambda = 0.353$, $P < 0.001$) morphometric parameters. However, these parameters were not significantly different between males and females (*Wilks'* $\lambda = 0.733$, $P = 0.155$).

Simple linear regressions revealed that body length was correlated with several parameters of insulation. There was a negative (slope = -0.0008) relationship between standard body length and blubber-to-core ratio (Fig. 5.4A; CIs = -0.00006, -0.0016). Body length was also positively (slope = 0.0378) correlated with desaturation index

(Fig. 5.4B; CIs = 0.0241, 0.0517) and negatively (slope = -1.7459) correlated with guard hair density (Fig. 5.4C; CIs = -2.6186, -0.8732).

5.5.6 Intra-species insulation patterns: leopard seals

Due to the small sample size, no MANOVA tests were performed to test in differences between age classes and sexes.

Simple linear regressions showed that body length had a negative (slope = -0.0018) relationship with blubber-to-core ratio (Fig. 5.5A; CIs = -0.0003, -0.0035). Body length was also positively (slope = 0.0203) correlated with desaturation index, but this relationship was not significant (Fig. 5.5B; CIs = -0.0028, 0.0434). Guard hair density did not change with body length (Fig. 5.5C; CIs = -2.1473, 2.1133).

5.5.7 Intra-species insulation patterns: Weddell seals

Sub-adults and adults displayed significantly different (*Wilks'* λ = 0.340, P = 0.013) morphometric parameters but males and females did not (*Wilks'* λ = 0.569, $P < 0.105$).

Body length correlated with insulation parameters in a pattern similar to crabeater and leopard seals. Larger seals had lower values of blubber-to-core ratio (Fig. 5.6A; slope = -0.0009), but this relationship was not significant (CIs = -0.0019, 0.0001), whereas desaturation index increased (slope = 0.0036) significantly with body length (Fig. 5.6B; CIs = 0.0130, 0.0589). Although guard hair density decreased with body length (Fig. 5.6C; slope = -0.5039), this relationship was not significant (CIs = -1.5629, 0.5550).

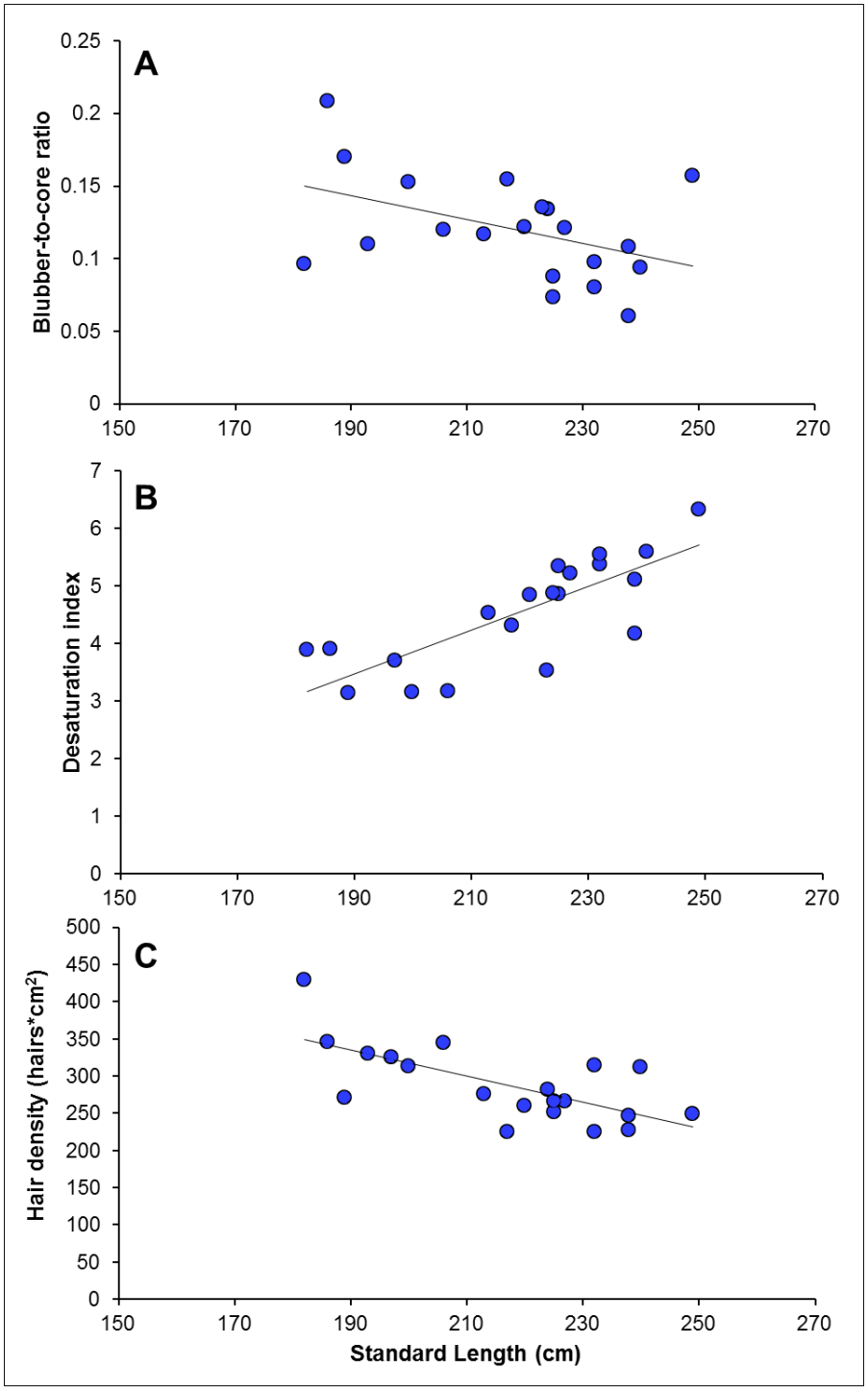


Figure 5.4. A) Blubber-to-core ratio, B) desaturation index, and C) guard hair density as a function of body length for crabeater seals. Body length was significantly correlated with blubber-to-core ratio ($y = -0.0008x + 0.29$), desaturation index ($y = 0.0379 - 3.73$), and guard hair density ($y = -1.7459x + 666.95$).

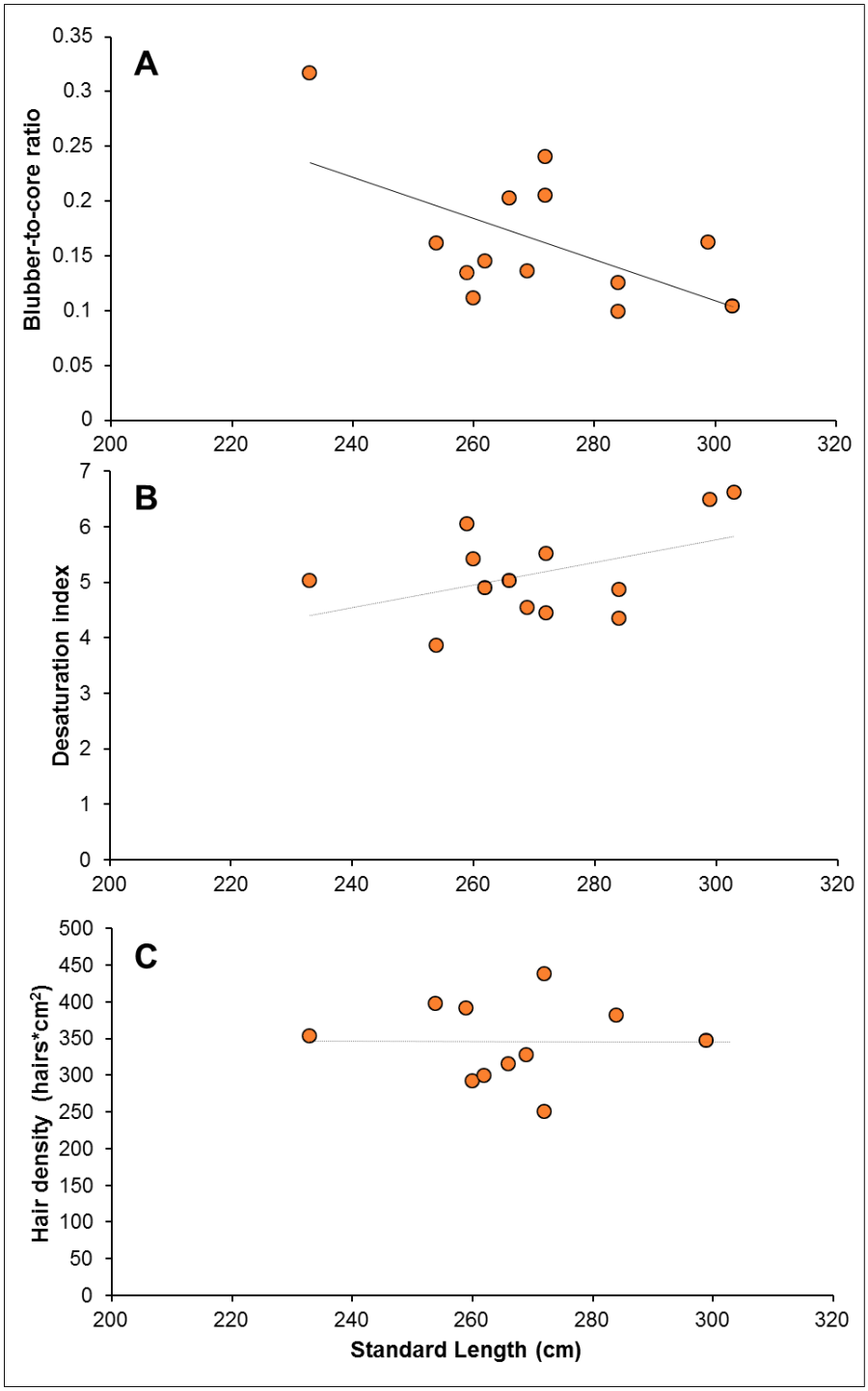


Figure 5.5. A) Blubber-to-core ratio, B) desaturation index, and C) guard hair density as a function of body length for leopard seals. Body length was significantly correlated with blubber-to-core ratio ($y = 0.0018x + 0.67$), but neither with desaturation index ($y = -0.0203x + 0.32$) nor with guard hair density ($y = 0.01702x + 349.79$).

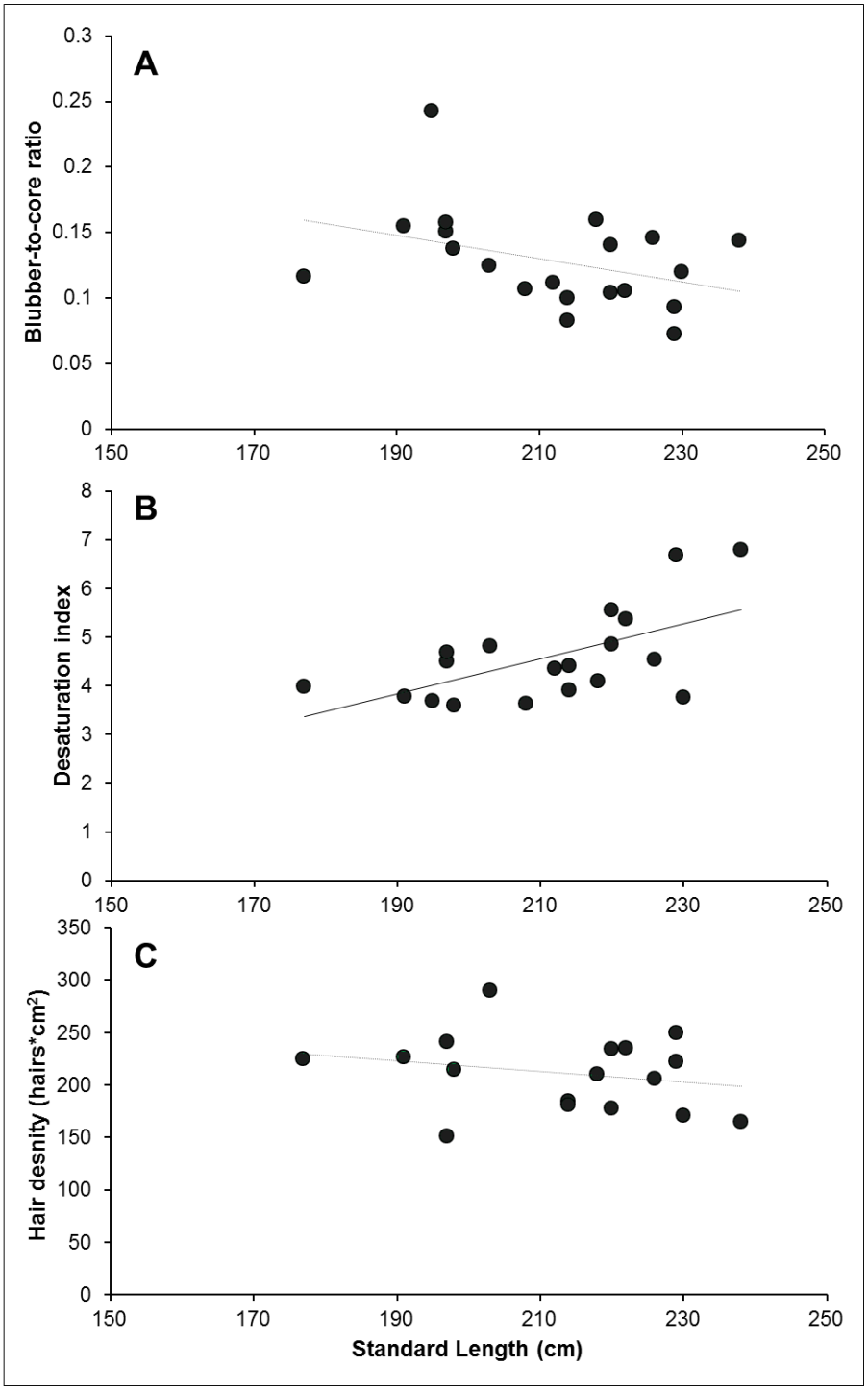


Figure 5.6. A) Blubber-to-core ratio, B) desaturation index, and C) guard hair density as a function of body length for Weddell seals. Body length was significantly correlated with desaturation index ($y = 0.03591x - 2.98$), but neither with blubber-to-core ratio ($y = -0.0008x + 0.31$), nor with guard hair density ($y = -0.5039x + 318.63$).

5.6 Discussion

5.6.1 Inter-species differences in thermal strategies

Overall, crabeater, leopard and Weddell seals display different physical characteristics with potential impact on thermoregulation. Although the PCA clearly separated the three species (Fig. 5.3), Weddell and crabeater seals display more similar characteristics and therefore are closer in the PC plot.

Leopard seals are different from crabeater and Weddell seals in several aspects of their morphology that could affect their resistance to cold temperatures. Leopard seals have the slimmest (highest fineness ratio) body form of the three species. Being a slim phocid (Van den Hoff et al., 2005) implies that they have higher surface area relative to the other two phocid species; therefore, they are potentially susceptible to higher rates of heat loss. Besides their slim body, leopard seals have larger flippers relative to their body length (limb ratio) than other seals. Extremities usually have very little insulation and offer a great surface area through which animals can expel heat. Due to these constraints, mammals living in cold environments, such as walrus, beluga *Delphinapterus leucas*, and bowhead whale (Reidenberg, 2007), tend to have shortened extremities, whereas the opposite is true in hot environments. According to the principles of Allen's rule (Allen, 1877), the body of the leopard seal would not be the ideal for cold environments. However this can be compensated by their larger body size, which can result in the generation of greater amounts of total body heat.

In addition, leopard seals have the lowest number of under furs per bundle, where only about half of their guard hairs are accompanied by under furs, and when they are present there is only one. A key role of under furs in pinnipeds is to maintain a layer of warm air between the skin and the guard hairs. In order to prevent water penetration, the ability of under furs to interlock with each other is crucial (Liwanag et al., 2012a), but

with such a reduced number of under furs it is very unlikely that the skin is being kept dry in water. Although it is known that in true seals, fur provides little insulation in water (Liwanag et al., 2012a), a reduced number of under furs could similarly reduce the effectiveness when hauling out on ice floes. This fact, added to the slim body and long extremities of leopard seals would make them more vulnerable than other seals to the vicissitudes of the extremely cold Antarctic.

Since body shape, appendages and fur layer of leopard seals seem to be susceptible to greater heat loss, the role of their blubber as an insulator becomes extremely important, and this was evident in this study. Their blubber layer is not just thicker (greater body-to-core ratio), but also able to resist lower temperatures and potentially more insulating (higher FA desaturation) compared to those of crabeater and Weddell seals. This improvement in the thermal properties of their blubber can then compensate the potentially higher heat loss rates due to their larger surface-to-volume ratio and poor fur layer.

These findings suggest that blubber is crucial for leopard seals to withstand freezing conditions. This implies that they need a relatively thick blubber with high FA desaturation. Since blubber is an energy reservoir made up of lipids obtained from excess of food intake (Castellini, 2009), leopard seals' thermoregulation may be very dependent on food availability, especially during winter. If the blubber layer is not thick enough and able to cool substantially their ability to keep warm could be compromised. Animals that have not developed a proper insulator by winter might be obligated to find warmer habitats. This could explain why the leopard seals further north, in mid-latitudes, tend to be younger animals (Gray et al., 2009); animals in poor health, as indicated by elevated serum protein fractions (Gray et al., 2005); and poor body condition (Gray et al., 2006; Gray et al., 2009). Although the majority of the leopard

seal population remains year round within the Antarctic pack ice (Meade et al., 2015; Rogers et al., 2005; Rogers et al., 2013; Southwell et al., 2012; Southwell et al., 2008), leopard seals are regular winter time visitors to subantarctic islands (Forcada and Robinson, 2006; Rogers, 2009). Lone leopard seals have been observed to remain year-round in mid-latitudes, along the southern continents, in Southern Chile (Acevedo et al., 2016; Acevedo and Martinez, 2012; Aguayo-Lobo et al., 2011); in Tasmania, Australia; and in Auckland, New Zealand. In comparison, crabeater and Weddell seals are rarely found in subantarctic or mid-latitude regions (Bengtson, 2009; Thomas and Terhune, 2009).

Alternatively, leopard seals' lifestyle may cause the generation of greater amounts of heat compared to other seals. Leopard seals feed at higher trophic levels, and are known to prey on other warm-blooded animals such as crabeater, Weddell and southern elephant seals (Hall-Aspland and Rogers, 2007), fur seals (Krause et al., 2015) and penguins (Rogers and Bryden, 1995). Leopard seals have been observed to conduct fast-swimming behaviours when in pursuit of penguins (Krause et al., 2015; Rogers and Bryden, 1995), this presumably would result in increased heat production.

Crabeater and Weddell seals have similar values of blubber-to-core ratio and FA desaturation; therefore their blubber layer may have similar thermal properties. However Weddell seals have the most rotund body, with the lowest fineness ratio and the shortest limbs, all of which have been indicated as some of the most important adaptations for aquatic life in extremely cold environments (Riedman, 1990). A body form ideal for life in the extreme cold may enable Weddell seals to withstand the coldest temperatures in Antarctica. Not surprisingly, the Weddell seal is the phocid with the most southerly distribution (Laws, 1977) and the only apex predator that remains year-round at high latitudes (~77ºS) in the Ross Sea (Burns and Kooyman, 2001)..

Besides, Weddell seals possess more under fur per bundle than the other seals. Air trapped in fur is susceptible to compression; therefore it may not be a good insulator in water (Liwanag et al., 2012b), especially in good divers such as Weddell seals (Kooyman, 1981). The ability to trap air next to the skin is likely more important when seals haul out on ice so that they can keep the cold air away from the skin surface (Castellini, 2009). For example, leopard seals inhabiting colder, more southerly regions (i.e., 68.4°S), are less likely to haul on windy days when the temperatures drop due to high wind-chill (Rogers and Bryden, 1997), whereas leopard seals inhabiting warmer, northerly regions (62.5°S) do not show this trend (Krause et al., 2015). The leopard seals low number of under furs is potentially poorer in-air insulating quality. Whereas water temperature in Southern Antarctica may not be very different than water in Northern Antarctica, air temperatures can be substantially different and much colder in southern regions.; therefore whereas a more southerly distribution in Antarctica may not imply great changes in water conditions compared to more northerly areas, the in-air temperatures can be much colder when the seals are hauled out of the water. In air the role of fur as an insulator can be crucial, and this, plus a rounder body form, may enable Weddell seals to occupy colder areas than leopard and crabeater seals.

5.6.2 Intra-species patterns: body length and changes in fatty acids

Larger animals have been found to be better adapted to cold environments. For instance, the lower critical temperature of larger mammals is lower than that of smaller mammals (Riek and Geiser, 2013). This means that small endotherms need to increase their metabolism to offset heat loss at higher temperatures than large endotherms. Larger arctic mammals, for example, can withstand decreasing temperatures with no need of increasing their heat production, whereas smaller arctic mammals do need to increase

their metabolic heat production to be able to resist low temperatures (Scholander et al., 1950b).

Due to the thermal constraints of having higher surface-to-volume ratios, smaller mammals lose proportionally more body heat than larger mammals (Pabst et al., 1999). Since small mammals should have more difficulties in keeping warm, I hypothesised that smaller seals would possess a more efficient insulating layer, either in the form of an improved fur or blubber. This was not evident, and in fact the opposite, in one of the blubber parameters measured: the FA desaturation of blubber. Smaller seals displayed lower desaturation indexes than larger seals. The desaturation index increased with body length in all three species, although it was not significant in the leopard seal, potentially due to the smaller sample size (Figs. 5.4B, 5.5B, 5.6B). A higher FA desaturation allows the skin to withstand lower temperatures and reduce heat loss through peripheral vasoconstriction. This may indicate that as seals grow, and presumably get older, their blubber becomes more important in insulation, withstanding lower temperatures without solidifying and thus reducing heat loss more effectively. In order to understand the causes of this correlation I investigated other thermal features that may accompany those changes in body mass.

5.6.7 Intra-species patterns: body length and changes in blubber thickness

Variations in body length are associated with other morphological changes in the organism. Although larger animals are able to carry more insulation (Hokkanen, 1990), the three species of Antarctic pack-ice seals studied here display smaller blubber-to-core ratios as they become larger, although this pattern was not significant in Weddell seals (Figs. 5.4A, 5.5A, 5.6A).

Blubber is made up of cells containing lipids which are supported in loose connective tissue (King, 1983). A thicker blubber layer can carry greater amounts of lipids, which could result in an increased thermal insulation (Pearson et al., 2014; Worthy and Edwards, 1990); so that less heat escapes through the blubber. Bottlenose dolphins, for example, can adapt to water temperature by changing their blubber thickness (Williams and Friedl, 1990). The decrease in blubber-to-core ratios in larger Antarctic pack-ice seals would, therefore, imply less insulation. However, due to the higher FA desaturation, this relatively thinner blubber core can effectively reduce heat loss and resist colder temperatures.

As discussed in Chapter 4, due to the difficulties of movement on land seals need to have relatively thinner blubber compared to cetaceans, but this still needs to be a good insulator and resist cold temperatures, which can be achieved, in part, by increasing the FA desaturation.

5.6.8 Intra-species patterns: body length and changes in fur density

Although blubber is the primary insulator in seals, they still possess fur, which is an extra insulating layer potentially more important when seals are out of the water. This study has shown that fur density is another aspect of seals' thermoregulation that changes with body length. As the seal grows, hairs become more widely spaced (King, 1983), so that smaller, potentially younger, animals have hairs that are more closely packed. This decrease in hair density was evident only in crabeater and Weddell seals, although it was not significant in the latter (Figs. 5.4C, 5.6C).

In pinnipeds, fur has undergone modifications for an aquatic lifestyle. Long hairs generate increased drag when swimming (Fish, 2000) therefore pinnipeds have

shortened hairs for better swimming performance. However, shorter hairs may facilitate the penetration of water into fur, losing insulation. This has also been solved through other adaptations. Aquatic mammals have developed oily furs, which help keep fur relatively impenetrable to water (Reidenberg, 2007). The hair of pinnipeds lack erector pili muscles, which serve to erect the hair (Berta et al., 2006). This may enhance the ability of the hairs to lie flat during submersion, further contributing to streamlining of the body by reducing drag forces (Berta et al., 2006) and also helping keep air trapped in between under furs and guard hairs (Liwanag et al., 2012a).

In Weddell and crabeater seals, the insulation provided by fur may be compromised due to their lower hair density when body length increases. Sparsely furred seals can have skin temperatures that approach those of the surrounding water (Elsner, 1999). When temperatures are very low, like in the Antarctic, the superficial tissues of seals need to remain fluid and avoid freezing; this can be achieved by lowering the tissues' solidifying point (Irving et al., 1957), which implies an increase in degree of FA desaturation. This has been reported to occur in the extremities of both pinnipeds (Samuel and Worthy, 2004) and arctic terrestrial mammals (Irving et al., 1957). Conversely, when fur is a good insulator and hence the skin temperatures are very near those of the body core, like in raccoon dogs, there is no need of an increase in FA desaturation (Käkëla and Hyvärinen, 1996a). In Chapter 4, I found an inverse correlation between fur density and FA desaturation in semi-aquatic mammals. Therefore, it is likely that the increase of FA desaturation with body mass in seals is, at least in part, a response to this decline in fur density.

Since body length is likely related to age, these findings suggest that as seals grow, they rely less on fur as insulator and more heavily on blubber. This concurs with Käkëla and Hyvärinen (1996a) who found that the insulation capacity of blubber develops with age.

Thus, older animals have lower skin temperatures and steeper temperature gradients across the blubber than younger animals. This thermal gradient from the cold skin to the warm body core allows a reduction of blood circulation near the surface, therefore reducing the amount of heat passed to the environment.

5.7 Conclusions

Although these three Antarctic pack-ice seals inhabit the same temporal and spatial environments, they may not have the same thermal needs as their thermal strategies differ. Leopard seals have slimmer bodies with larger projecting appendages compared to crabeater and Weddell seals. This may increase their heat loss due to their higher surface-to-volume ratios. Besides, they have fewer number of under furs, which may not be sufficient to maintain a warm layer of air between skin and fur. Therefore, their blubber plays a key role in thermoregulation and is potentially more important in the leopard than in crabeater and Weddell seals. Leopard seals have the thickest blubber layers of all three species with the highest desaturation indexes. On the other hand, Weddell seals have a rotund body shape with short flippers, which is ideal for life in extremely cold conditions. They also possess a potentially more thermally-efficient fur due to a higher number of under furs per bundle, which is beneficial for remaining warm while the seal is hauled out of the water.

Insulation patterns change with body length in Antarctic pack-ice seals. As seals grow, their blubber-to-core ratios become smaller and their fur becomes sparser in some species, but this would not mean poorer insulation because the blubber has increased FA desaturation, which allows them to withstand lower temperatures without solidifying. This suggests that potentially seals develop a more efficient blubber layer

with age, an insulator that is not disproportionally thick, but more efficient in reducing heat loss.

CHAPTER 6

General Discussion

6.1 The use of blubber fatty acids as trophic markers

Fatty acids can leave strong signals in the biochemical structure of consumers (Brett et al., 2016) and many studies have demonstrated the validity of using FAs for dietary inference. In this study, I have conducted dietary analyses in two Antarctic predators, the leopard and the crabeater seal, and my results support the idea that FAs are good trophic markers. These seals are sympatric species sharing some of their prey types; however they have different foraging strategies. The leopard seal is a generalist, preying mainly on krill during summer in the Western Antarctic peninsula (Casaux et al., 2009), but also taking other prey types such as penguins and young seals (Hall-Aspland and Rogers, 2007; Rogers and Bryden, 1995). The crabeater seal, on the other hand, is a specialist consumer; therefore and ideal model species to study the FA signal of prey in their blubber FAs.

As predicted, the influence of prey was clearer in the blubber FAs of the crabeater seal (Chapter 3), which was not surprising as its blubber is made up of dietary FAs coming mostly from one single prey type. In the leopard seal (Chapter 2), the consumption of more than one prey, each with different FA composition, makes the FA signal of prey less evident, but still recognizable.

Predators feeding on a variety of prey are common in ecological studies, where FA analysis can be challenging as blubber FAs will correspond to a mixture of FAs coming from a variety of sources. In Chapter 2, although the inner layer aligned more close together with krill FAs, the resemblance was not absolutely clear. Some of the FAs driving the segregation between prey and predator were endogenous or partially endogenous FAs, which could have been avoided if only dietary FAs were used. In Chapter 3, I used only dietary FAs (PUFAs and certain MUFAs) to determine diet and the results suggest that this method can predict prey types with better resolution than

using the whole array of FAs. This is due to some FAs not being of dietary origin but have been modified or synthesised intrinsically. As seen in Chapter 4 and 5, MUFAs and SFAs will be modified according to the consumer's physiological needs. Therefore, the use of only dietary FAs is ideal to trace diet.

There are still some limitations to the use of FA analysis as trophic markers. There exists considerable uncertainty regarding "the trophic modification" of individual FAs, when consumers incorporate dietary resources into their own tissues (Brett et al., 2016). The FAs of the prey consumed could be directly used, or accumulated with little or no modification. This will be particularly important when animals are experiencing major physiological demands. The Weddell seal, for example, mobilise FAs selectively during the lactation period (Wheatley et al., 2007). Therefore, this technique should be used cautiously because there may be specific FAs that are mobilised differentially according to the physiological state of the animal. It is important to ensure that animals are in a positive energy state (i.e.: accumulating energy) and not mobilising great amounts of FAs (i.e.: fasting) since some of their FAs will be depleted and others will remain in their blubber, which would result in a biased interpretation of FA composition.

In order to overcome some of these limitations, the combined use of FAs with other biochemical methods such as stable isotopes has been proposed (Dalsgaard et al., 2003). Similar to FA analysis, the isotopic composition of an animal's tissues can be used to elucidate its diet, since isotopic values are correlated with those of its prey items (Gannes et al., 1997; Hückstädt et al., 2012). The combined use of these methods is promising and has shown to be a more accurate technique to determine diet (e.g.: (Herman et al., 2005; Hooker et al., 2001; Kharlamenko et al., 2001).

6.2 Implications of fatty acid stratification for dietary studies

The stratification of FAs across the blubber layer has implications for sampling methods in the field. The collection of the inner blubber core requires the chemical sedation or physical restraint of research animals whereas collection of the outer blubber samples can be done remotely by biopsy darting of free-ranging animals. In the case of large cetaceans, the outer layer is the only sample that can be typically obtained. Therefore, it is important to understand how this FA stratification will affect dietary or physiological studies.

Given the differences in FA content between inner and outer layers, the use of either of these samples for dietary studies may not provide the same results. In Chapter 2, I have shown that when comparing predator and prey FAs, the inner layer is closer to the prey, suggesting that if this layer is available, it should be used to infer diet. In fact, the use of the inner layer has been recommended by several authors (Grahl-Nielsen, 2009; Grahl-Nielsen et al., 2011; Olsen and Grahl-Nielsen, 2003; Skoglund et al., 2010). However, the collection of the inner layer can be difficult, especially in free-ranging large cetaceans. In Chapter 3, I compared and found no difference between the diet predicted for crabeater seals when using FA signatures of dietary origin from outer and inner blubber layers. Although the FAs within the outer layer are more susceptible to changes related to their functional (potentially thermoregulatory) role, the exclusion of FAs prone to modification (SFAs and MUFAs) would allow this outer blubber layer to be used to obtain a more accurate dietary inference. Therefore, this could be a good alternative when working with animals difficult to handle in the field.

The factors behind the stratification of FAs within the blubber of marine mammals are not yet fully understood. I have provided the FA composition of two Antarctic phocids, for which no information of blubber FAs had been reported. Studies like these,

providing FA composition of different blubber layers, can be very helpful in order to understand the mechanisms associated with the necessity of having different FA contents across different depths of the blubber layer. This information can then be used for future ecological, comparative and/or physiological studies that can help us understand the big picture.

These two seal species showed a stratification pattern similar to other marine mammals. This was not surprising and I have largely discussed in Chapter 1 and 2 how this stratification supports the idea of different physiological roles of the blubber layers. However, although this stratification pattern is ubiquitous among marine mammals, the degree of this stratification is not. As discussed in Chapter 3, otariids have been found to have the smallest degrees of stratification whereas cetaceans seem to have the highest levels; however the reasons are not fully understood. It has been suggested that this is related to thermoregulation, as some cetaceans living in colder environments have more stratified blubber than others inhabiting warmer waters (Koopman, 2007). However, there are few comparative studies focused on understanding why blubber is more stratified in some animals compared to others. In order to understand this phenomenon, we need to look at the bigger picture. There are challenges, and caveats, in conducting a comparative analysis to understand the FA stratification in marine mammal blubber, for example where some studies report the FA composition of the whole blubber core, others report values for the outer section of the blubber only.

The FA stratification of marine mammal blubber is strongly related to the desaturation of the FAs. The typical stratification pattern corresponds mainly to an increase of MUFAs and decrease of SFAs in the outer layer compared to the inner layer. This suggests that SFAs are being desaturated (turned into MUFAs) at a higher rate in the outer layer. In Chapter 4, I investigated whether this desaturation was caused by thermal

acclimatization. The use of desaturation index allowed me to perform a more robust analysis, providing information that could help understand not only the drivers of the FA stratification but also the functional role of the outer blubber layer.

6.3 The role of non-dietary fatty acids

In Chapter 4, I showed that the desaturation of FAs occurs actively in the outer layer of marine mammals, which is evidenced by the higher desaturation index of fully-aquatic mammals compared to terrestrial mammals, and by the plasticity of semi-aquatic mammals in modifying FAs according to their thermal habitats. This is the first study that analyses the effect of a physical trait (hair density) along with external factors (latitude and environment) on non-dietary FAs across mammalian taxa. My results indicate that the desaturation of FAs in the adipose tissues of mammals is presumably driven by environment and may have been an important adaptation of mammals during the move from land to water. MUFAs and SFAs are susceptible to modification within an organism, and this can be especially important if the animal lives in a cold environment. Semi-aquatic mammals will be more prone to modify their endogenous FAs in different environments; therefore this should be taken into consideration when performing dietary studies.

As seen in Chapter 4, semi-aquatic mammals actively modify their FAs depending on the geographical area they live in. This can have implications for dietary studies. For example, when using FAs to study two populations of semi-aquatic mammals inhabiting different thermal habitats, it should be expected that those animals living in the colder environment may have higher amounts of MUFAs and smaller amounts of SFAs in their blubber. If this is accounted for, the dietary analysis could be interpreted more accurately.

I also showed that semi-aquatic mammals will have different FA desaturation depending on their fur density. As FAs are potentially modified depending on the tissues temperature, MUFAs are expected to be more abundant when tissues are colder. Thus, if we want to infer diet using FAs of sympatric populations of fur seals compared to sea lions that inhabit a cold environment, we are likely to see greater amounts of MUFAs in the outer layer of sea lions compared to the fur seals. This is because sea lions do not have fur with a high insulative quality so that their outer blubber layer will be at a lower temperature than that of the fur seals. The fur seals on the other hand, have fur with high quality insulative properties so that their outer blubber layer is maintained at a typical higher body temperature. Therefore, even if the fur seals and the sea lions fed on the same prey species, there would be differences in their FAs signatures, differences caused by the different physiological demands upon the FAs within their blubber.

The drivers of the metabolism of MUFAs and SFAs are still under debate. There are many factors that can affect the desaturation of FAs and I show here that the effect of thermal acclimatization might be just one of them. To calculate the desaturation index of FAs, I used only those FAs that are endogenous or intrinsically synthesised; however, some FAs can be partially endogenous and partially dietary. This is a limitation to this type of analysis. Budge et al. (2006) states that in monogastric predators, the greatest contributor to adipose tissue FA composition is direct deposition of FA from diet. It can be argued that diet can still have an influence in the desaturation of FAs. A recent study by Abbott et al. (2012) shows that rats fed 12 diets showed changes in their MUFA content. In this study, when rats were fed fat that was high in MUFAs the adipose FA composition became increasingly higher in MUFAs, and vice versa. However, there are other examples where diet has had little influence on MUFAs and SFAs. For instance,

in humans PUFAs show a close relationship between dietary intake and adipose tissue whereas SFAs and MUFAs are less closely correlated (Garland et al., 1998). Radio-labelling studies showed that grey seals transformed SFAs into MUFAs because the radio-labelled palmitic acid 16:0 they were fed was modified into 16:1 in their blubber (Budge et al., 2004). Other authors agree that MUFAs and SFAs are not expected to reflect diet intake (Baylin et al., 2002). In Chapter 4, there could still be a dietary influence in the FA desaturation of mammals, however the meta-analysis approach I conducted allows us to see that, when examined more broadly, there is an interesting influence of thermoregulation on FAs and that this is a fertile area to investigate further.

In Chapter 5, I examined the influence of thermoregulation on FAs desaturation in closer detail. I quantified the desaturation index of the FAs in the blubber of three closely related seal species (the Antarctic pack ice seals) that live in the same thermal habitat but have different diets and foraging behaviour. I show that there were no significant differences in the FA desaturation between the three species. Although the seal species had other differences with potential impacts on their thermoregulatory mechanisms, their desaturation index was not different even when they have different diets. The Weddell seal eats mainly fish and squids, while the leopard seal takes penguins and krill, and the crabeater seal feeds exclusively on krill. The fact that desaturation index was not different supports the hypothesis of an effect of thermal acclimatization on the desaturation of FAs in the outer layer of mammals, as discussed in Chapter 4.

I showed that body length, potentially strongly related to age, is another factor that influenced the FA composition within a species. This has been reported for other marine mammals, such as killer whales *Orcinus orca* (Herman et al., 2009), steller sea lions *Eumetopias jubatus* (Beck et al., 2007) and harbour porpoises (Koopman et al., 1996),

where differences in certain FAs have been found to be related to age. Therefore, when studying diet in a population this should be taken into account. There could be indeed differences in diet related to different food intake between younger and older animals, but there could also be differences in FAs related to long-term accumulation.

6.4 Conclusions

The use of FAs as trophic markers are promising as a method to study foraging ecology, and this is supported by my studies on leopard and crabeater seals. Since MUFAs and SFAs are more prone to intrinsic modification due to other factors apart from diet, I recommend the use a subset of dietary FAs for dietary studies. Thus, either the inner or outer layer could be used, as the FA signal of the prey will be present in both layers and could be more easily traceable using dietary FAs in the outer layer. When comparing populations from different thermal habitats, it is important to consider that populations living in colder environments are likely to have higher amounts of MUFAs than those in warmer habitats, particularly in semi-aquatic mammals. These differences should not be attributed to different diets alone.

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Appendix 1

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Vertical fatty acid composition in the blubber of leopard seals and the implications for dietary analysis

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ABSTRACT

The analysis of blubber fatty acids (FAs) is a useful tool to infer diet of mammals that live in remote regions where year-round studies are difficult. The FA may not be distributed uniformly within the blubber, which can have implications for dietary predictive studies. The aim of this study was to determine the FA composition in the blubber core of the Antarctic leopard seal, *Hydrurga leptonyx*, and evaluate the potential implications of FA stratification for dietary analysis. The blubber cores of 24 seals were sub-sectioned into outer, middle and inner layers and their FA were compared to those of their potential prey species. A vertical variation in FA composition was found across the whole blubber core of the leopard seal. 17 FAs were found at greater than trace amounts (>0.5%) across all samples and the most abundant were: C18:1ω9, C16:1, C22:6ω3, C16:0 and C18:1ω7, which accounted for approximately 70% of the total FA. Almost all FAs had a continuous gradient through the blubber. Principal Component Analysis confirmed separation between inner and outer layers while the middle layer was a transition. The stratification of the leopard seal blubber was similar to the general pattern observed in a variety of marine species: monounsaturated FA (MUFA) dominated the three layers being more abundant in the outer layer, polyunsaturated (PUFA) and saturated FA (SFA) were more abundant in the inner layer. Polyunsaturated FAs are of dietary origin and SFAs are chemically inert so they can be used as a long-term reserve, which suggest that the inner layer is the site of deposition of the FA obtained from diet. The influence of prey on the composition of the leopard seals' blubber was clearer in the inner layer, although neither outer nor inner layers exactly matched the FA of the potential prey. This suggests that there are other components influencing the FA composition of this predator; therefore, in order to carry out dietary analysis it is important to consider the stratification of blubber and to use the inner layer, where the influence of diet is more evident. This has significant implications for sampling methods in the field.

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1. Introduction

The blubber of a marine mammal plays an important role in a variety of functions such as energy reserve, buoyancy control, streamlining and thermal insulation (Castellini et al., 2009; Samuel and Worthy, 2004). The fatty acids (FAs) making up this adipose tissue are mainly from dietary origin (Grahl-Nielsen et al., 2010); for this reason the FA composition of blubber has attracted considerable scientific interest, because of its potential applicability for studies of foraging ecology. Fatty acid analysis can provide a long-term indication of diet history (Bradshaw et al., 2003; Dahl et al., 2000); therefore it can be used to obtain more complete data on diet composition than traditional methods, such as scat and stomach analyses. Although these methods are useful to determine overall trends (Hoberecht, 2006), they have known biases and limitations associated with incomplete consumption of prey items, gut passage rate and differential degradation of prey remains (Arnould et al., 2005).

Fatty acid analysis is based on the principle that particular FAs are present in prey and can be transferred mainly unmodified into the blubber of the predator (Iverson, 1993). This occurs because while animals produce a limited number of FA, most FAs are synthesised by phytoplankton at low trophic levels, so that the presence of specific compounds can be attributed only to diet (Bromaghin et al., 2012; Budge et al., 2008).

The FA composition of blubber, however, is not exactly identical to that of the diet (Cooper et al., 2005; Grahl-Nielsen, 2009) since it may be regulated by other factors (Grahl-Nielsen and Mjaavatten, 1991). Researches have described that many marine mammals exhibit at least two distinct blubber layers: the outer layer, located under the epidermis; and the inner layer, located just above the muscle (Best et al., 2003; Lambert et al., 2013; Samuel and Worthy, 2004). They

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may also exhibit a third layer which is usually a transition between inner and outer blubber layers (Aguilar and Borrell, 1990). This variation, or stratification, in the composition of blubber indicates that some components of blubber are synthesised independently of diet and may have different functions (Wheatley et al., 2007), which complicates the investigation of foraging ecology through this method.

The inner blubber layer is thought to be metabolically more active and dietary FAs are most likely deposited in this layer rather than in the outer layer (Best et al., 2003; Budge et al., 2008; Herman et al., 2005). On the other hand, it has been suggested that the outer layer may have another physiological role (Grahl-Nielsen et al., 2011) and differs from the diet (Grahl-Nielsen, 2009). Therefore, before using the blubber for obtaining dietary information, it is important to determine the species-specific FA composition through the blubber.

Different studies have taken different approaches to infer diet; some use either the outer component only (Herman et al., 2005; Waugh et al., 2012), or a section across the whole blubber (Bradshaw et al., 2003; Meynier et al., 2008; Newland et al., 2009) while other studies recommend the use of the inner component (Grahl-Nielsen et al., 2005; Olsen and Grahl-Nielsen, 2003; Skoglund et al., 2010). This has implications for sampling as collection of the inner blubber core requires the chemical sedation or physical restraint of research animals whereas collection of the outer blubber samples (just under the skin) can be done remotely by biopsy darting of free-ranging animals.

The leopard seal, *Hydrurga leptonyx* (Blainville, 1820), is an ideal model to study their foraging ecology via FA analysis, as they live in the remote Antarctic pack ice where long-term dietary studies are very difficult to carry out. They have a dispersed distribution (Forcada et al., 2012; Rogers et al., 2013; Southwell et al., 2008), individuals travel widely (Meade et al., 2015; Rogers et al., 2005) and haul out on the drifting ice floes (Rogers and Bryden, 1997; Southwell et al., 2003; Rogers et al., 2013) making year-round studies difficult. Their feeding ecology has been determined by different means including direct hunting observations (Ainley et al., 2005; Hiruki et al., 1999; Penney and Lowry, 1967; Rogers and Bryden, 1995), stomach contents (Siniff and Stone, 1985), scats (Casaux et al., 2009; Green and William, 1986; Hall-Aspland and Rogers, 2004, 2007; Rogers and Bryden, 1995; Walker et al., 1998) and stable isotope (Hall-Aspland et al., 2005a,b) analyses. This study will identify initially whether the blubber of the leopard seal is stratified by establishing the vertical variation in FA composition across whole blubber cores. The influence of stratification of FAs on dietary studies will be tested by comparing how FA composition of the inner and outer blubber layers perform in inferring diet and trophic level from the same individuals.

2. Materials and methods

2.1. Sample collection

In total, 24 leopard seals, 9 females and 15 males, were sampled off the Danco Coast, Western Antarctic Peninsula (64°09′ S 60°57′ W) during the austral summer (February) of 2008 and 2009. Seals hauled out on sea ice were immobilised using a Tele-inject air gun darting system using tiletamine/zolazepam (Higgins et al., 2002). Following immobilisation, sex, standard length (SL straight line nose to tail) and pectoral girth (PG) were recorded and 8 mm diameter biopsies containing whole cores of blubber (from the skin to the muscle layer) were collected from the mid-dorsal surface. All individuals were adults and the body length averaged 280.8 ± 18.3 mm in females and 269.3 ± 19.1 mm in males. In the laboratory, the blubber cores were separated into three sections (inner, middle and outer layers) as shown in Fig. 1. Each sample was stored in airtight vials and frozen at −20 °C for further analyses. The immobilisation and sampling of leopard seals in the Antarctic Specially Protected Area No. 134 was approved by the Dirección Nacional del Antártico, Buenos Aires, Argentina, and performed according to the SCAR Code of Conduct for Animal Experiments under UNSW Animal Care and Ethics Committee (Protocols 08/103B and 11/112 A).

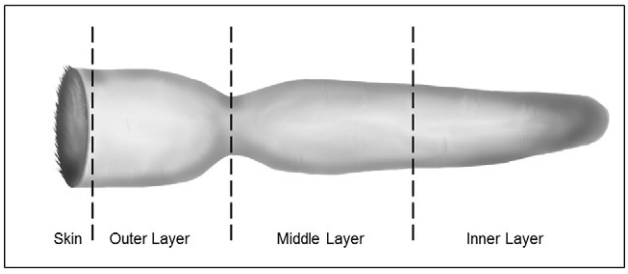

Fig. 1. Sectioning of the blubber core. The outer layer was taken between the skin and the narrowest part of the blubber column. The remaining sample was divided into two sections: the middle layer (the closest to the outer layer) and the inner layer (the closest to the muscle).

2.2. Fatty acid analysis

Total lipid was extracted following a modified Folch et al. (1957) method. Approximately 0.3 g–0.5 g of blubber was weighed and placed in 9 ml of 2:1 chloroform: methanol with 50 mg/L of butylated hydroxytoluene in test tubes with Teflon caps. Samples were mashed manually with a glass homogeniser until thin and transparent, then vortexed for 20s, and allowed to soak overnight at 4 °C. To remove protein precipitates the homogenate was filtered into Teflon screw cap glass tubes, 2 ml of water were added and the whole mixture was agitated and then allowed to separate into two phases. The lower phase containing lipids was carefully collected by siphoning/pasture pipette and placed in Teflon lined screw cap tubes. The solvent (chloroform) was evaporated from lipid under nitrogen stream to avoid lipid oxidation.

Fatty acid methyl esters (FAMEs) were prepared directly from the extracted lipid, which was dissolved in 1.5 ml of boron trifluoride (10% in methanol) and 1.5 ml of toluene. The solution was capped under nitrogen and heated at 50 °C overnight. Esters were extracted into hexane and stored in vial tubes for gas chromatography analysis.

Gas chromatography analyses were performed with Agilent 6850 Series GC System (Agilent Technologies, Santa Clara, CA, USA) equipped with a flame ionisation detector. FAMEs were analysed using a 100 m long fused carbon-silica capillary column (SP 2560 Column). The flow rate of the hydrogen carrier gas was set at an initial flow rate of 2.7 ml/min, at a pressure of 200 kPa. The injector and detector port temperatures were set at 260 °C. The column oven was initially held at 140 °C for 5 min, then increased 4 °C/min and then maintained at a temperature of 235 °C. The total run time per cycle was 35 min. Peak areas and retention times were calculated (ChemStation Software, Rev. B.03.01; Agilent Technologies), and FAME were identified by comparison of retention times with a range of standards. The concentrations of individual FA in each sample were converted to percentage contributions of the total FA.

2.3. Data analyses

Fatty acids present in trace amounts (<0.5%) were excluded from statistical analyses. Thus, the number of FA was reduced to 17. The largest FA proportion was more than 2000 times greater than the smallest FA proportion, so all values (x) were arcsin-transformed ($x' = \arcsin\sqrt{x}$) in order to reduce the heterogeneity of variance among groups. Normality and homogeneity of variance were checked with normality plots and with plots of residuals versus fitted values, respectively.

Due to the multivariate nature of FA profile data, Principal Component Analysis (PCA) was used to investigate patterns in FA association among the different individuals. In this manner the 17 variables could be described in two dimensions. Multivariate analysis of variance

(MANOVA) was then carried out on the PC scores with gender, year, and blubber layers as factors.

A stratification index was calculated by subtracting the percentage in the inner layer from percentage in the outer layer and dividing the difference by mean of totals for outer and inner layers (Olsen and Grahl-Nielsen, 2003). This index was calculated in order to determine the degree of stratification in the blubber.

A correlation between body condition and inner layer FA was tested. Body condition was assessed using two condition indices: Smirnov index and fineness ratio (Van der Hoff et al., 2005) were derived from standard body length (*SL*) and girth (*PG*) measurements.

Fineness ratio (*FR*) is an index to measure streamlining and hydrodynamic performance. Assuming that, when supported by water the seals body would have a roughly circular cross-section it is calculated as the standard body length (*SL*) divided by its maximum diameter (D_{ma}):

$$FR = SL \div D_{ma}$$

where maximum diameter was calculated from the girth (*PG*) measurement using the equation:

$$D_{max} = PG_{max} \div \pi$$

All statistical tests have an α level of statistical significance of 0.05 (SPSS Release 17.0, SPSS 2008). Where not identified variability around the mean is standard deviation.

2.4. Potential prey fatty acids

In order to test whether the use of inner or outer layers would lead to the same predictions of diet, a PCA for leopard seal blubber layers and potential prey FA composition was plotted. Additionally, their trophic level was investigated by using FA ratios of vaccenic acid/oleic acid (C18:1ω7/C18:1ω9) and eicosapentaenoic acid (EPA)/docosahexaenoic acid (DHA) (C20:5ω3/C22:6ω3).

Data of prey species was collated from published literature, which included species of fish (Connan et al., 2010; Lea et al., 2002; Mayzaud et al., 2011; Raclot et al., 1998), krill (Alonzo et al., 2005; Cripps et al., 1999; Phleger et al., 2002; Polito et al., 2012) and penguins (Speake et al., 1999; Tierney et al., 2008).

3. Results

3.1. Fatty acid stratification

Although 46 FAs were originally identified, only 17 (comprising 89.34% of total) were found consistently in all blubber layer samples and in proportions greater than 0.5%. The most abundant FAs, accounting for approximately 70% of total FA, were: C18:1ω9 (19–27%), C16:1 (10–17%), C22:6ω3 (6–9%), C16:0 (6–10%), and C18:1ω7 (7–8%) (Fig. 2). The FA profiles of the three layers were dominated by monounsaturated FAs (MUFAs) (40–55%), followed by polyunsaturated FAs (PUFAs) (20–30%) and with smaller proportions of saturated FAs (SFAs) (12–19%) (Table 1, Fig. 3).

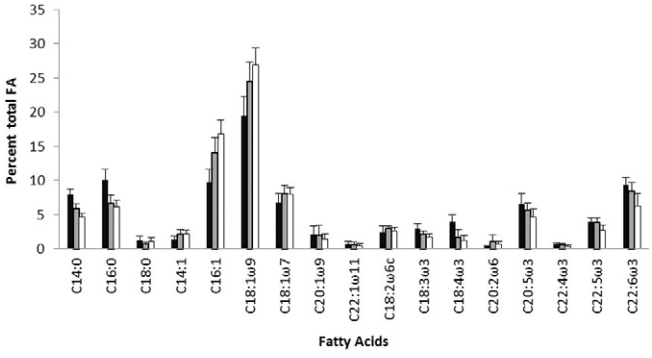

Fig. 2. Proportion of fatty acids across blubber layers. Relative amounts (percent total) of 17 fatty acids (FAs) in the inner (black), middle (grey) and outer (white) blubber layers of leopard seals, *H. leptonyx* (n = 24).

Monounsaturated FAs were present at higher proportions in the outer blubber layer (55.7 ± 4.2%) decreasing to 40.1 ± 5.3% in the inner layer. On the other hand, SFAs and PUFAs are present at higher proportions in the inner layer (19.3 ± 2.7% and 30.4 ± 3.8%, respectively) rather than in the outer layer (11.9 ± 1.6% and 20.1 ± 5.0%, respectively).

The first three components derived from the PCA accounted for 67% of the variation (PC1 43%, PC2 13%, PC3 11%) in FA composition among the samples. The bivariate plot (Fig. 4) shows inner and outer layers as separated groups, while the middle layer sits between them. The principal FA driving the segregation of the PC1 were the positive Eigen values for C18:4ω3, C18:3ω3, C14:0 and C22:6ω3 (more abundant in the inner layer) and the negative Eigen values for C16:1, C18:1ω9 and C18:1ω7 (more abundant in the outer layer). A MANOVA was carried out on the 3 PC scores and it confirmed the significant differences between blubber layers (Wilks' $\lambda = 0.154$, $P < 0.001$). Fig. 4 also shows a group of seals, whose inner layer was separated from the others and was rather sitting with the middle layer group. This segregation of leopard seals inner blubber was not caused by differences in body condition of the seals, since there was no correlation between the fineness ratio ($t_{18} = 0.42$, $P = 0.67$) or Smirnov index ($t_{18} = 1.23$, $P = 0.23$), and the first PC for inner layer FA across the 24 seals.

The inner-outer difference per individual FA is represented by the stratification index (Fig. 5). The mean overall FA stratification index was 0.44 ± 0.22 and the maximum reached an absolute value of 1.11 for stearidonic acid (C18:4ω3). While SFAs were more abundant in the inner layer, MUFAs were enriched in the outer layer. Most PUFAs showed an inner-layer trend, except C18:2ω6c and C20:2ω6, which were in greater amounts in the outer layer.

There were no significant inter-sexual (Wilks' $\lambda = 0.961$, $P = 0.482$) or inter-yearly (Wilks' $\lambda = 0.955$, $P = 0.413$) differences.

3.2. Fatty acids and implications for dietary analysis

From the principal component analysis comparing leopard seals to their potential prey species (Fig. 6), the PC1 values indicate segregation between prey and predator, which is influenced by higher amounts of C16:0 and C20:5ω3 in prey species and higher amounts of C18:1ω9 and C16:1 in leopard seals. The inner layer aligned more closely with prey species, compared to the outer layer. The second PC reveals similarity between fish and penguins, with higher amounts of C18:0 and C20:1ω9, and between leopard seals and krill species, due to the influence of positive Eigen values for C18:4ω3 and C14:0.

Fig. 7 reveals that leopard seals, penguins and most fish species have low vaccenic acid/oleic acid ratios whereas most krill species have higher ratios. For leopard seals, penguins and most fish species, EPA/DHA ratios are low; but krill species show a wide range of values.

4. Discussion

4.1. Stratification

Significant variations were observed in the FA composition between the outer and inner blubber layers, however rather than being discrete layers the FA composition changed as a gradient from the outer, middle to the inner layer through the blubber core. Blubber stratification has been reported for several phocid species including: harbour seals, *Phoca vitulina* (Andersen et al., 2004); southern elephant seals, *Mirounga leonina* (Best et al., 2003); Baikal seals, *Phoca sibirica*; ringed seals, *Pusa hispida* (Grahl-Nielsen et al., 2005; Strandberg et al., 2008);

Table 1
Fatty acid profiles* (per cent total FA) of inner, middle and outer blubber layers from leopard seals (n = 24).

	2008						2009					
	Female			Male			Female			Male		
	IL	ML	OL	IL	ML	OL	IL	ML	OL	IL	ML	OL
C14:0	8.21	6.12	4.99	7.41	5.67	4.77	7.97	5.98	4.49	8.25	5.75	4.61
C16:0	10.42	6.78	6.88	9.38	6.24	4.93	10.40	7.07	6.46	10.33	6.60	6.86
C18:0	1.22	0.89	1.41	1.12	0.88	0.62	1.58	0.67	0.96	1.38	0.73	1.49
Σ SFA	19.85	10.34	13.28	17.91	7.99	10.32	19.95	10.98	11.91	19.96	11.21	12.96
C14:1	1.50	2.04	2.17	1.42	2.39	2.63	1.31	1.76	1.73	1.34	2.28	1.97
C16:1	10.00	13.50	15.91	10.62	14.36	17.90	9.58	14.55	16.77	8.70	13.91	15.99
C18:1ω9	20.44	23.35	27.10	19.96	24.67	27.17	18.55	22.83	25.66	18.84	25.90	27.36
C18:1ω7	6.73	8.69	8.20	7.05	7.92	7.72	6.29	8.58	7.86	6.64	7.42	8.13
C20:1ω9	1.60	1.16	1.39	2.77	2.91	1.79	1.59	2.00	1.32	2.04	1.79	1.17
C22:1ω11	0.47	0.66	0.32	0.68	0.75	0.61	0.69	0.30	0.26	0.82	0.57	0.35
Σ MUFA	40.74	37.06	55.08	42.50	33.13	57.83	38.01	40.01	53.60	38.37	44.47	54.96
C18:2ω6	2.04	3.05	2.69	2.21	2.92	2.30	2.94	2.80	2.86	2.68	3.11	2.66
C18:3ω3	3.18	2.22	1.66	2.89	2.22	2.01	2.71	1.99	1.55	3.03	2.09	1.48
C18:4ω3	3.38	2.10	1.26	3.82	1.84	1.79	4.30	1.08	0.84	4.06	1.75	0.77
C20:2ω6	0.27	0.67	0.34	0.37	0.73	0.66	0.44	1.74	0.83	0.39	1.00	0.66
C20:5ω3	5.93	5.52	4.00	6.97	5.35	5.05	7.13	5.99	5.09	6.04	5.72	4.14
C22:4ω3	0.55	0.75	0.31	0.77	0.65	0.53	0.86	0.53	0.38	0.76	0.62	0.25
C22:5ω3	3.99	4.00	2.42	3.75	3.62	3.02	4.34	4.33	2.97	3.97	3.63	2.40
C22:6ω3	9.30	8.94	5.62	8.74	8.11	6.90	9.99	9.02	6.75	9.69	8.29	5.50
Σ PUFA	28.66	20.43	18.30	29.52	15.90	22.27	32.71	21.99	21.29	30.61	22.47	17.87

* Only those FAs contributing >0.5% of the total are shown.

Weddell seals, *Leptonychotes weddellii* (Wheatley et al., 2007); and harp seals, *Pagophilus groenlandicus* (Grahl-Nielsen et al., 2011). Winter and Nunn (1950) reported FA composition of the whole blubber core from three sites (the belly, back and neck) on the body of a single leopard seal. Similar FA compositions were reported between these three sites, but only four individual FAs were identified and stratification of the blubber core was not considered.

The stratification of blubber is attributed to the different roles that the layers provide, either related with thermoregulation for the outer layer and dietary processes for the inner layer (Arnould et al., 2005; Best et al., 2003; Koopman et al., 1996).

Saturated FAs were more abundant in the leopard seals' inner blubber layers than in the outer layers. In otariids there is little stratification of SFA across the blubber (Arnould et al., 2005; Lambert et al., 2013) but SFA stratification is pronounced in other phocids (Grahl-Nielsen et al., 2005; Strandberg et al., 2008), in the walrus (Skoglund et al., 2010) and in cetaceans (Budge et al., 2008; Olsen and Grahl-Nielsen, 2003). Saturated FAs are thought to be important for long-term energy storage (Arriola et al., 2013) since they are comparatively chemically inert (Christie, 2003). In addition, having the highest melting point compared to the other groups of FA (Christie, 2003), the decreasing amount of SFAs towards the outer layer is reasonable as greater amounts of SFAs in the outer layer would make it very rigid, particularly at low temperatures, which would be unfavourable for movement and streamlining.

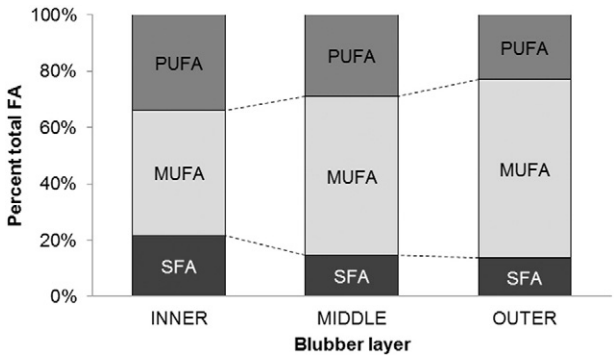

Fig. 3. Fatty acid groups across blubber layers. Mean proportion of polyunsaturated (PUFA), monounsaturated (MUFA), and saturated (SFA) fatty acids from the inner, middle and outer blubber layers for leopard seals, *H. leptonyx* (n = 24).

Leopard seals have higher quantities of MUFAs across all layers, but there are significantly more MUFAs in the outer blubber layer. This is similar to the MUFA distribution patterns across the blubber of other phocid seals, the harp (Grahl-Nielsen et al., 2011), ringed (Strandberg et al., 2008) and southern elephant (Best et al., 2003) seals as well as the otariid: the New Zealand sea lion, *Phocarctos hookeri* (Lambert et al., 2013). It has been suggested that because MUFAs have a lower melting point, relative to their saturated counterparts (Christie, 2003; Rustan and Drevon, 2005), their presence in the outer layer is important to maintain softness and fluidity under the skin and to help reduce heat loss from the body (Best et al., 2003). Moreover, in the case of cold-water inhabiting species, the temperature of the outer layer could be extremely low, in ringed seals, for example, while the outer layer temperature is 5 °C, the muscle temperature is 36 °C (Käkelä and Hyvärinen, 1996). Low temperatures may limit enzymatic reactions (Koopman, 2007) which could affect dietary processes ion the outer layer.

Polyunsaturated FAs are more abundant in the leopard seals' inner blubber layer, which is not common across other seals, but has been reported for several cetacean species. They are in higher abundance in the

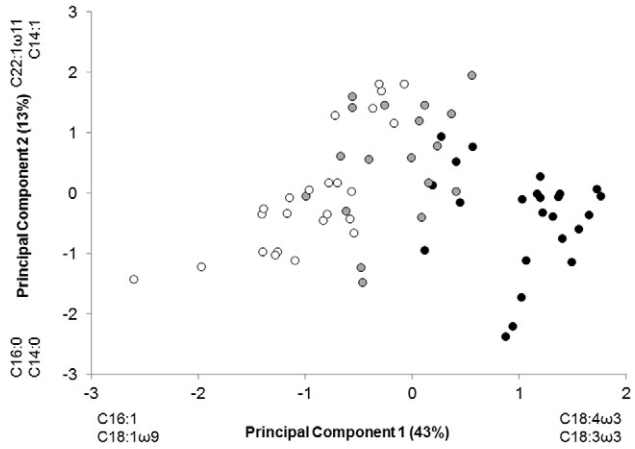

Fig. 4. Vertical variation of fatty acids across leopard seal blubber. Principal component plot for the inner (black circles), middle (grey circles) and outer (white circles) blubber layers of leopard seals, *H. leptonyx* (n = 24).

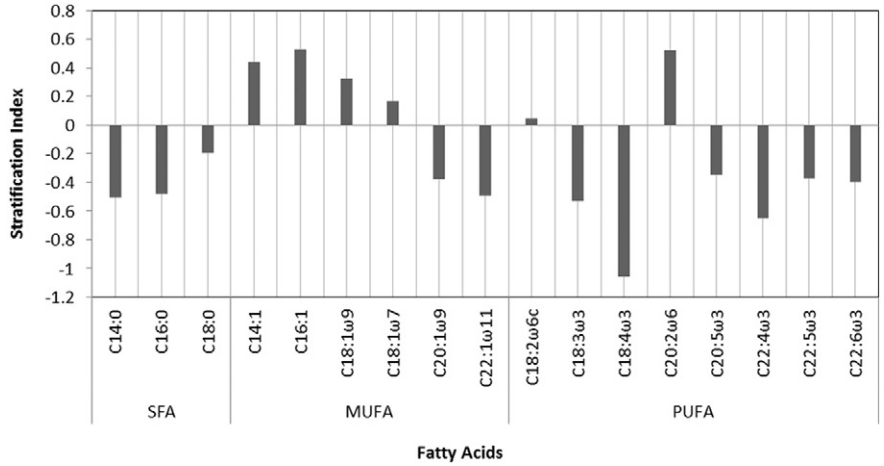

Fig. 5. Stratification Index for fatty acids of leopard seal blubber (n = 24). Positive values represent a greater amount of the fatty acid in the outer layer and negative values indicate a greater presence in the inner layer.

inner blubber layer of dusky dolphins, *Lagenorhynchus obscurus* (Grahl-Nielsen et al., 2010), short-beaked common dolphins (Smith and Worthy, 2006), harbour porpoises, *Phocoena phocoena* (Koopman et al., 1996), minke (Olsen and Grahl-Nielsen, 2003) and fin whales (Ruchonnet et al., 2006). Polyunsaturated FAs are similar in quantities across the inner and outer layer of the phocids: Weddell seals (Wheatley et al., 2007), ringed seals (Grahl-Nielsen et al., 2005; Strandberg et al., 2008); and the otariids: the cape fur seal, *Arctocephalus pusillus* (Arnould et al., 2005) and New Zealand sea lion (Lambert et al., 2013); the Atlantic walrus, *Odobenus rosmarus* (Skoglund et al., 2010); and the cetacean: the bowhead whale, *Balaena mysticetus* (Budge et al., 2008).

Higher presence of PUFAs in the inner blubber layer of the leopard seal may be due to their prevalence in the marine environment and the fact that they are usually of a dietary origin (Hoberecht, 2006; Samuel and Worthy, 2004). Several PUFAs are essential FAs, which must be acquired through the diet (Arriola et al., 2013; Liwanag et al., 2012). They are produced by phytoplankton and are tightly conserved with little catabolism in predators (Dahl et al., 2000; Hoberecht, 2006). As PUFAs are susceptible to oxidative deterioration or autoxidation (Christie, 2003), they are rapidly available from the inner layer as a fuel source (Arriola et al., 2013). Despite this, the differences in PUFA distribution between marine mammals can be explained by distinct deposition and mobilisation of FAs through the depth of the blubber, which will also depend on their nutritional state.

Overall, the stratification index of the leopard seals' FA shows similar values to those found on other phocids (Grahl-Nielsen et al., 2005, 2011; Strandberg et al., 2008, 2011) but larger than those reported for otariids (Arnould et al., 2005; Lambert et al., 2013). This means that phocids have blubber with higher vertical variation than otariids. Pinnipeds in general, use a combination of blubber and fur as a mechanism of insulation (Liwanag et al., 2012), but phocids blubber as thermal insulator, since their fur has poor insulating properties when wet (Kvadsheim and Aarseth, 2002). Therefore, the higher level of blubber stratification in phocids could be a consequence of its better specialisation as an insulator: the outer layer may have differentiated to play an exclusive thermoregulatory role while the inner layer is the energy reserve ready to be utilised when the animal needs it.

4.2. Fatty acids and implications for dietary analyses

The two most abundant FAs (C18:1ω9 and C16:1) present in leopard seal's blubber are ubiquitous to marine mammals. They have been recorded in the highest proportions for phocids (Andersen et al., 2004; Grahl-Nielsen et al., 2005; Strandberg et al., 2008; Wheatley et al.,

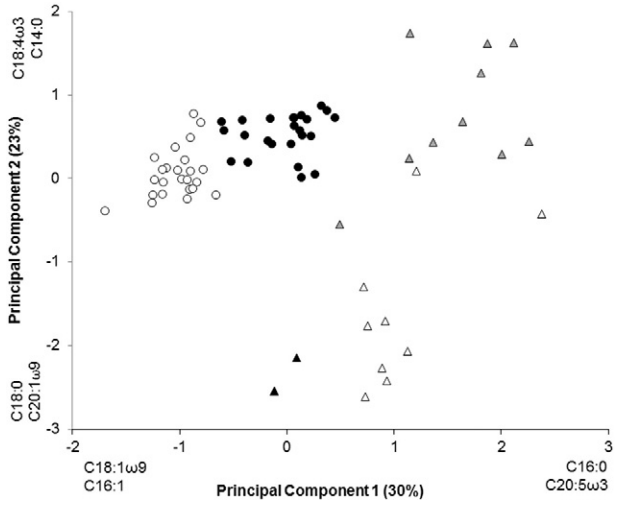

Fig. 6. Dietary inference from fatty acids. Principal Component plot of leopard seals inner (black circles) and outer (white circles) blubber layers (n = 24) and their potential prey species: fish (white triangles), krill (grey triangles) and penguins (black triangles).

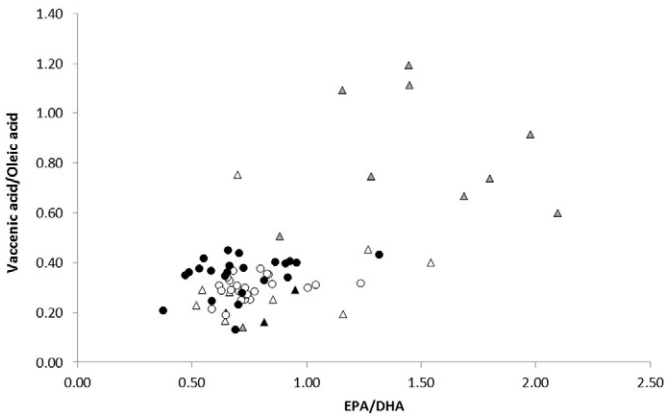

Fig. 7. Trophic level analysis. Vaccenic acid/oleic acid and eicosapentaenoic acid (EPA)/docosahexaenoic acid (DHA) ratios of leopard seals' inner (black circles) and outer (white circles) blubber layers, krill (grey triangles; Alonzo et al., 2005; Cripps et al., 1999; Phleger et al., 2002; Polito et al., 2012), fish (white triangles; Connan et al., 2010; Lea et al., 2002; Mayzaud et al., 2011; Raclot et al., 1998) and penguins (black triangles; Speake et al., 1999; Tierney et al., 2008).

2007), otariids (Beck et al., 2007; Iverson et al., 1997; Lambert et al., 2013; Rosen and Tollit, 2012) and cetaceans (Budge et al., 2008; Quérouil et al., 2013; Samuel and Worthy, 2004; Waugh et al., 2012). They are considered endogenous FAs (Herman et al., 2005) which do not have a dietary origin (Raclot et al., 1998), although it has also been suggested that high levels of these FAs may be consistent with a carnivorous diet (Phleger et al., 2002).

Docosahexaenoic acid (C22:6ω3) was the third most abundant FA present in leopard seal's blubber, which is similar to other phocids (Arriola et al., 2013; Grahl-Nielsen et al., 2005; West et al., 1979), otariids (Arnould et al., 2005; Beck et al., 2007; Iverson et al., 1997), Atlantic walrus (Skoglund et al., 2010), and some cetaceans (Budge et al., 2008; Quérouil et al., 2013; Ruchonnet et al., 2006; Smith and Worthy, 2006). This is an exogenous dietary FA (Herman et al., 2005) and hence a likely biomarker for the assessment of long-term dietary intakes (Raclot et al., 1998). Moreover, the higher presence of DHA in the inner stratus supports the thesis of a dietary role of this layer. This FA is characteristic of krill species (Tierney et al., 2008); therefore, the higher presence of DHA in leopard seals compared to other Antarctic seals such as southern elephant (Best et al., 2003) and Weddell (Wheatley et al., 2007) seals suggests that leopard seals are incorporating higher levels of krill in its diet. The other FA linked to krill intake is docosapentaenoic acid (C22:5ω3) (Tierney et al., 2008) which is also more abundant in the leopard seals than reported in the other Antarctic seals (Best et al., 2003; Wheatley et al., 2007).

Gondoic acid (C20:1ω9) was in low (~2%) quantities in the leopard seal inner blubber compared to quantities in other phocid seals; such as the southern elephant (Best et al., 2003), harbour (Andersen et al., 2004) and ringed (Grahl-Nielsen et al., 2005; Strandberg et al., 2008) seals; where it represents from 10 to 15% of the dietary FA in the blubber. This FA has a dietary origin and is characteristic of many teleost species but it is found in very low levels in krill (Iverson et al., 1997). This may indicate a low intake of fish in leopard seals, unlike the other seals that may be preying on fish more heavily.

When the FAs of the leopard seals were compared with those of their potential prey species (from the literature), there was no definitive indication that the seals were feeding on any of the prey items included in this study, irrespective of the blubber sampling site (inner or outer blubber layer). This study supports others (Best et al., 2003; Grahl-Nielsen et al., 2011; Waugh et al., 2012) where FAs of the predator have not matched exactly with those of their prey. A close alignment between predator and prey FAs was not anticipated here as the FA results from prey were not collected at the same sampling site but prey FAs were obtained from the literature, an approach typical of other FA dietary studies (Bradshaw et al., 2003; Newland et al., 2009). The aim of this study was to identify how the inner and outer layers performed, relative to one another, in predicting dietary items. Whereas the FAs of the outer layer were clustered together and were not closely aligned with the FA of any of the potential prey species, the FA of the inner blubber layer aligned more closely with FA of krill, but only on the PC2 axis (Fig. 6), which was driven by higher amounts of C18:4ω3 and C14:0, which are FA from dietary origin (Raclot et al., 1998). Leopard seals in this region are known to feed on krill, from scat analysis (Casaux et al., 2009), conferring with the prediction from the FA values of the innermost layer.

FAs of either the inner or outer blubber layer were equally valid trophic predictors for the leopard seal. The ratio of vaccenic acid to oleic acid is used frequently to estimate the degree of carnivory (lower values) versus herbivory (Stubing and Hagen, 2003). The analysis of vaccenic acid/oleic acid and EPA/DHA ratios shows that the leopard seals inner and outer blubber layers align together along with other Antarctic predators: most fish species and penguins (Fig. 7). The wide range of krill EPA/DHA ratios indicates that different krill species derive energy from either a diet of diatom (high ratio) or flagellate (low ratio) origin (Phleger et al., 2002). The similar trophic level of leopard seals, fish and penguins suggests that there is no a predator–prey relationship. Krill species are in lower trophic levels, which can be indicative of leopard seals preying on them. Identifying that the ratios of vaccenic acid/oleic acid and EPA/DHA were similar between the outer and inner leopard seal blubber layers has significant implications for sampling methods. To collect samples from the inner blubber core (closest to the muscle) requires the chemical sedation or physical restraint of a research animal in order to collect a total blubber core whereas the outer blubber layer (just under the skin) can be collected remotely by biopsy dart from free-ranging animals.

5. Conclusions

This study provides the first description of FA composition of the leopard seal. The study of feeding ecology through FA analysis has become a valuable tool to study feeding behaviour of animals living in remote regions, like the Antarctic, where year-round studies are difficult. As shown in this study, the considerable variation in FA composition across the blubber structure of the leopard seal needs to be taken into account. The ability to use samples from the outer blubber layer for FA analysis has significant implications for sampling methods. The outer blubber layer can be collected remotely by biopsy dart from free-ranging animals and it is commonly the most feasible way of sampling, especially in animals that cannot be sedated, such as cetaceans. To collect samples from the inner blubber core requires the chemical sedation or physical restraint of the research animal in order to collect a total blubber core. For the leopard seal we recommend the use of the innermost blubber layer for diet prediction, as the influence of diet in the outer layer is significantly reduced. However, even though the inner layer will provide more accurate results on diet inference, this may not exactly indicate the prey items consumed by the predator, as predator's metabolism will influence their FA composition. This biochemical technique should be used in complement with others, such as stable isotopes analysis, to minimise the biases produced by the animal's metabolism.

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Appendix 2

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Blubber fatty acid composition and stratification in the crabeater seal, *Lobodon carcinophaga*

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ABSTRACT

The study of blubber fatty acids (FAs) has attracted increasing scientific interest due mainly to its potential use as trophic markers. This is possible because most FAs are transferred unmodified from the prey to the blubber of the predator. Additionally, FAs have also been used to understand other aspects of the biology of marine mammals, including thermoregulation and reproductive stage. The aim of this study is to determine the FA composition of the blubber of the crabeater seal, which has not been reported before, and identify stratification of FAs across the blubber layer. Seals were captured in the western Antarctic Peninsula during the austral summer of 2015 and a whole blubber core (down to the muscle layer) sample was collected. The blubber core was sub-sectioned into inner and outer layers and analysed for FAs. The FA composition of crabeater seals was similar to other marine mammals, with high proportions of 18:1ω9, 16:1ω7, and 16:0. These FAs are endogenous, that is, they can be readily synthesised by mammals. However, the high proportions of 22:6ω3 and 20:5ω3 are unusual among other marine mammals. These FAs are potentially reflecting the diet of crabeater seals, as they have a dietary origin and are known to be abundant in krill, the main prey item of this predator. Inner and outer layers were significantly different indicating a stratification of blubber FAs. The pattern of stratification is ubiquitous in marine mammals, with monounsaturated FAs increasing towards the outer layer, and saturated and polyunsaturated FAs increasing towards the inner layer. This difference between inner and outer layers may indicate that they have different metabolic roles. Because most dietary FAs were more abundant in the inner layer, this is likely to be the section where the dietary process takes place. The higher proportions of monounsaturated FAs in the outer layer suggest that this section of the blubber plays a more functional role, probably related to thermoregulation, as these FAs prevent rigidity and improve fluidity, which is particularly important in cold environments.

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1. Introduction

Blubber is a special adaptation of the skin of marine mammals that has multiple functions related to energy storage, buoyancy, thermal insulation, and streamlining (Iverson, 2009; Ryg et al., 1988). Due to the variety of roles played by this tissue, the study of its biochemical composition, specifically the fatty acid (FA) signature, can provide insights into several aspects of the life of an animal.

One of the most important applications of the study of blubber FA composition is the inference of food items in the diet of a predator. This method is based on the principle that FAs are transferred almost unmodified from the prey to the blubber of the predator (Iverson, 1993; Iverson et al., 2004). This is due to the inability of mammals to synthesise a major part of their FAs; therefore they are assimilated through the diet and stored as energy reserve (Cook and McMAster, 2002; Iverson, 1993). Consequently, the dietary origin of these FAs could be tracked and thus provide information on the food items consumed (Dalsgaard et al., 2003).

Fatty acids can provide information on the dietary intake over a longer period of time compared to only the most recent feeding provided by traditional stomach and scat content analyses (Dalsgaard et al., 2003; Iverson, 1993). For example, Bradshaw et al. (2003) detected differences in food consumption between winter and summer, and between foraging regions using FAs from the blubber of southern elephant seals, *Mirounga leonina*. Similarly, Kirsch et al. (2000) found that blubber FAs reflected changes in the diet of captive harp seals, *Pagophilus groenlandicus*. Consequently, this method has become scientifically attractive and is particularly useful to study more cryptic mammals, or where the collection of samples is difficult to carry out during the whole year.

Additionally, FAs have been used to study other aspects of the physiology of marine mammals related to their thermal habitats. The effect of environmental temperature on FA composition of tissues has been demonstrated in carp fish (Tiku et al., 1996), and there is some evidence that suggests that a similar effect may occur in the blubber of marine

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mammals. Käkëla and Hyvärinen (1996), for example, suggested that the type of FAs found in the cold extremities of mammals corresponds to an adaptation to low temperature. Similarly, Samuel and Worthy (2004) found differences in FAs of bottlenose dolphins between winter and summer that can be attributed to environmental conditions. Therefore, although this area of study has been explored to a lesser extent than dietary applications, it can potentially provide valuable information on the capacity of marine mammals to adapt to different climatic conditions.

Other less explored uses of FA analysis in marine mammals include the relationships between FAs and age (Beck et al., 2007; Herman et al., 2009; Koopman et al., 1996), sex (Beck et al., 2007), reproductive state (Kellar et al., 2006; Samuel and Worthy, 2004) and diving behaviour (Koopman, 2007). Therefore, FA analysis is a promising area of study that can have multiple applications when investigating different aspects of marine mammals' life history.

Our study species, the crabeater seal *Lobodon carcinophaga*, is the most abundant phocid seal in the world (Siniff, 1991) but one of the least studied of the Antarctic ecosystem (Casaux et al., 2009; Gales et al., 2004). They are year-round residents of the Antarctic pack ice (Laws, 1985) where they usually aggregate in large groups (Gales et al., 2004; Laws, 1985). Crabeater seals have lobed teeth (Siniff, 1991) that are specialised for consumption of Antarctic krill, which is their main prey item (Forcada et al., 2012; Hückstädt et al., 2012; Laws, 1985). >3 million crabeater seals consume over 12 million tonnes of krill each year, approximately 17% of the krill standing stock (Forcada et al., 2012). Their blubber reaches its maximum thickness (~5 cm) in late summer/early autumn and maintains this level throughout the winter (Laws et al., 2003; McDonald et al., 2008; Øritsland, 1970). After the pupping and breeding season that takes place in spring (Siniff et al., 1979) lipid reserves are utilised causing a reduced blubber thickness (McDonald et al., 2008; Siniff, 1991) which reaches its minimum (~2–3 cm) during early summer (Castellini et al., 2009; Laws et al., 2003). In this study, seals were captured after the breeding season in midsummer, where seals are expected to be in a positive energy state and accumulating lipids in their blubber.

The first aim of this study was to examine the blubber FA composition of the crabeater seal. Among the Antarctic phocid seals, the FA composition of blubber has been reported for southern elephant seals (Best et al., 2003); leopard seals, *Hydrurga leptonyx* (Guerrero et al., 2016); and female Weddell seals, *Leptonychotes weddellii* (Wheatley et al., 2007); but not yet for ross seals, *Ommatophoca rossii*; nor for crabeater seals.

The second aim of this study is to determine the degree of stratification of FAs in the blubber of crabeater seals. Most marine mammals have been reported to have a stratified blubber (Best et al., 2003; Grahl-Nielsen et al., 2011; Guerrero et al., 2016; Quérouil et al., 2013), which implies that the outer blubber layer (under the skin) has a different FA composition compared to the inner layer (above the muscle layer). This feature of blubber can have implications for dietary studies (Lambert et al., 2013); therefore, it is necessary to assess the existence of FA stratification prior to further analyses. To do this, the FA composition of crabeater seals in outer and inner blubber layers were analysed separately.

Crabeater seal females tend to be slightly heavier and longer than males of similar age (McDonald et al., 2008; Laws et al., 2003), which could have an effect on their blubber properties. Similarly, some aspects of blubber are known to experience changes with age (Herman et al., 2009; Pearson et al., 2014). Therefore, we will identify potential differences between sexes and age classes.

The new data made available in this study can be used in future ecological studies. FA composition can be useful to understand the drivers of the stratification of FAs in marine mammals' blubber and can provide valuable insights into not only predator-prey relationships but the flow of energy within the whole ecosystem.

2. Materials and methods

2.1. Sample collection

From mid-January to mid-February of 2015, 20 crabeater seals (11 females and 9 males, Table 1) were sampled off the Danco Coast, Western Antarctic Peninsula (64°09′ S 60°57′ W). Seals were immobilised with tiletamine/zolazepam (Higgins et al., 2002) using a Tele-inject air gun darting system. Sex and morphometric measures were recorded, and an 8 mm diameter biopsy sample was collected from the mid-dorsal surface. Biopsy samples contained fur, skin and a whole blubber core (down to the muscle layer). The immobilisation and sampling of crabeater seals in the Antarctic Specially Protected Area No. 134 was approved by the Dirección Nacional del Antártico, Buenos Aires, Argentina; and performed according to the SCAR Code of Conduct for Animal Experiments under UNSW Animal Care and Ethics Committee (Protocol 15/55A).

In the laboratory, skin, fur and blubber were separated. The blubber cores were measured with a calliper and divided into two sections: the inner (approx. 8 mm from the blubber adjacent to the muscle) and the outer layer (approx. 10 mm from the blubber adjacent to the skin). Each sample was stored in an airtight vial and frozen at −20 °C for further analyses.

2.2. Fatty acid analysis

Total lipid of crabeater seal blubber and whole krill samples was extracted following a modified Folch et al. (1957) method (Budge et al., 2006). Briefly, samples were extracted with 2:1 chloroform: methanol containing 0.01% of butylated hydroxytoluene, washed in a salt solution, centrifuged, dried over anhydrous sodium sulphate and evaporated under nitrogen.

Fatty acid methyl esters (FAMEs) were prepared with an acidic transesterification procedure, using H_2SO_4 in methanol (Budge et al., 2006). Esters were extracted into hexane at a concentration of 50 mg/mL and stored in vial tubes for gas chromatography analysis.

Gas chromatography analyses were performed with Agilent 7890A Series GC System (Agilent Technologies, U.S.A.) equipped with a flame ionization detector. FAMEs were analysed using a 30 m × 0.25 mm flexible fused silica column coated with 50% cyanopropyl polysiloxane (0.25 μm film thickness, DB-23, Agilent Technologies, U.S.A.). The injector and detector port temperatures were held at a constant temperature of 250 °C. The helium split flow was set at a rate of 100 mL/min and the

Table 1
Parameters measured in the 20 crabeater seals sampled.

Seal ID	Sex	Age class	Body mass (kg)	Blubber thickness (cm)	Body length (m)
CS1501	Male	Sub-adult	226	2.5	2.13
CS1502	Male	Adult	246.4	2.6	2.27
CS1503	Male	Adult	259.6	2.6	2.20
CS1504	Female	Adult	–	3.4	2.49
CS1505	Female	Sub-adult	199	3.0	2.00
CS1507	Male	Sub-adult	196.6	2.3	1.93
CS1508	Male	Adult	271.4	2.2	2.25
CS1509	Male	Adult	278.6	2.5	2.38
CS1510	Female	Adult	266.6	1.4	2.38
CS1511	Female	Sub-adult	216.6	2.6	2.06
CS1512	Male	Adult	260	1.8	2.32
CS1513	Female	Adult	266.2	1.6	2.25
CS1514	Female	Adult	269	2.1	2.40
CS1515	Female	Sub-adult	159.8	3.2	1.89
CS1516	Male	Adult	226.6	3.1	2.17
CS1517	Male	Adult	271.6	2.3	2.32
CS1518	Female	Sub-adult	146.4	1.8	1.82
CS1519	Female	Adult	267.4	3.0	2.24
CS1520	Female	Sub-adult	163.6	3.6	1.86
CS1521	Male	Adult	240.4	2.8	2.23

carrier gas flow (He) was 1 mL/min. The flow rates of air and hydrogen to the detector were 450 mL/min and 45 mL/min, respectively. The column oven was initially held at 153 °C for 2 min, and then increased at a rate of 2.3 °C/min to 174 °C. That temperature was maintained for 0.2 min and then ramped at 2.5 °C to 210 °C. This final temperature was held for 2 min. Thus, the total run time per cycle was 28 min. Peak areas and retention times were calculated (ChemStation Software, Rev. B.03.01; Agilent Technologies), and FAMEs were identified by comparison of retention times with a range of standards. The concentrations of individual FAs in each sample were converted to percentage contributions of the total FAs.

2.3. Data analyses

Only FAs present in amounts > 0.5% were included in these analyses, since trace amounts are more likely to contribute noise (Grahl-Nielsen et al., 2011). Thus, the number of FAs was reduced to 17. Due to the multivariate nature of FA data, Principal Component Analysis (PCA) was used to determine patterns in the FA composition of crabeater seals. In this manner the 17 variables corresponding to each FA identified could be described in two dimensions. Due to proportional FA data does not have a normal distribution, this was log transformed according to the formula recommended by Aitchison (1986), using 18:0 as reference as suggested by Budge et al. (2006). Multivariate analysis of variance (MANOVA) was then applied to the first 2 PC scores to test for differences between blubber layers, age classes and sexes.

To further investigate the patterns in stratification of FAs, paired sample *t*-tests were applied to test for differences in FA types (saturated FAs, SFAs; monounsaturated FAs, MUFAs; and polyunsaturated FAs, PUFAs) between outer and inner layers. In addition, a stratification index (SI) was calculated according to the following equation by Olsen and Grahl-Nielsen (2003):

$$SI = \frac{(F_o - F_i)}{\left(\frac{F_o + F_i}{2.}\right)}$$

where F_o is the percentage concentration of each FA in the outer layer and F_i is the concentration in the inner layer. Thus, positive values of SI represent a greater amount of the FA in the outer layer and negative values indicate a greater presence in the inner layer.

Simple linear regressions were used to test whether FA stratification index was correlated with body mass or blubber thickness. All statistical tests were performed in R (version 3.1.1) and significance was deemed with an $\alpha < 0.05$ or when confidence intervals (CIs) extracted from slope values did not overlap 0. Values are reported as mean ± standard deviation (SD), unless otherwise noted.

3. Results

3.1. Fatty acid composition

Blubber cores had a thickness of 2.52 ± 0.60 cm (Table 1). Only 17 FAs were found in amounts > 0.5% across all blubber samples (Table 2). The most abundant FAs, in decreasing order, were: 18:1ω9, 16:1ω7, 22:6ω3, 20:5ω3, 16:0, 18:1ω7 and 14:0, which accounted for approximately 75% of total FAs (Fig. 1).

Overall, the most abundant type of FA in the blubber of crabeater seals was MUFA (Table 2, Fig. 2), which comprised 40.95 ± 4.56% of total FA in the inner layer, and 53.31 ± 3.42% in the outer blubber layer. The proportion of MUFAs was significantly different between layers ($t_{19} = 9.782$, $P < 0.001$). PUFAs were the second most abundant type of FA, being significantly ($t_{19} = -8.947$, $P < 0.001$) more abundant in the inner layer with 35.07 ± 2.74% compared to the outer layer with 26.16 ± 3.95%. Similarly, SFAs were significantly ($t_{19} = -7.008$, $P < 0.001$) more abundant in the inner than in the outer layer, with 17.03 ± 2.37% and 12.67 ± 1.99% respectively.

Table 2
Fatty acid profiles* (per cent total FA + SD) of the inner and outer blubber layers of crabeater seals (n = 20).

	Inner Layer	Outer Layer
14:0	6.40 ± 0.60	5.00 ± 0.85
16:0	8.66 ± 1.83	5.93 ± 1.31
17:0	0.90 ± 0.47	0.76 ± 0.39
18:0	1.07 ± 0.27	0.97 ± 0.23
Σ SFA	**17.03 ± 2.37**	**12.67 ± 1.99**
14:1ω5	1.62 ± 0.58	2.56 ± 0.47
16:1ω7	12.50 ± 2.66	18.40 ± 1.34
18:1ω9	18.84 ± 2.22	24.05 ± 2.80
18:1ω7	6.41 ± 0.43	7.21 ± 0.48
20:1ω9	1.57 ± 0.45	1.09 ± 0.27
Σ MUFA	**40.95 ± 4.56**	**53.31 ± 3.42**
18:2ω6c	2.01 ± 0.25	1.98 ± 0.17
18:3ω3	3.08 ± 0.74	1.68 ± 0.48
18:4ω3	1.56 ± 0.62	1.31 ± 0.39
22:2ω6	1.37 ± 0.21	1.18 ± 0.24
20:5ω3	9.81 ± 2.29	7.50 ± 1.98
22:4ω3	0.97 ± 0.19	0.59 ± 0.17
22:5ω3	4.44 ± 1.04	3.52 ± 0.92
22:6ω3	11.83 ± 1.52	8.41 ± 1.80
Σ PUFA	**35.07 ± 2.74**	**26.16 ± 3.95**

* Only those FAs contributing >0.5% of the total are shown.

3.2. Fatty acid stratification

The stratification index indicates the inner-outer differences per individual FA (Fig. 3). A negative stratification index was found in all SFAs and PUFAs, indicating their greater abundance in the inner layer. On the other hand, most MUFAs had a positive stratification index due to their greater dominance in the outer layer. The only MUFA with a negative index was the long-chain 20:1ω9, which was enriched in the inner layer. The mean overall stratification index was 0.27 ± 0.16 and the most stratified FA was 18:4ω3, with an index of −0.58. Although there was a slight positive relationship, the stratification index was not significantly correlated with either body mass (slope = 0.008; CIs = −0.0004, 0.0019) or blubber thickness (slope = 0.0051; CIs = −0.0034, 0.0136).

Principal component analysis, including all 17 individual FAs, confirmed separation between blubber layers. Fig. 4 shows inner and outer layers as separated groups. PC1 explained 46% of the variation while PC2 explained 25%. The separation of blubber layers is evident on the PC2, where the negative Eigenvalues of 18:4ω3 and 22:4ω3 and positive Eigenvalues of 14:1 and 16:1 are driving most of this segregation. A MANOVA applied to the first 2 PC scores confirmed that inner and outer layers are significantly different (*Wilks'* $\lambda = 0.381$, $P < 0.001$). However, there were no significant differences between sexes (*Wilks'* $\lambda = 0.976$, $P = 0.683$), or age classes (*Wilks'* $\lambda = 0.973$, $P = 0.664$), or interaction between any of these variables.

4. Discussion

4.1. Fatty acid composition of blubber

Crabeater seals possess a FA composition similar to other marine mammal top predators. Large proportions of 18:1ω9, 16:1ω7, 16:0 and 18:1ω7 have been reported for other phocids, including Weddell seals (Wheatley et al., 2007); leopard seals (Guerrero et al., 2016); southern elephant seals (Best et al., 2003); and ringed seals, *Pusa hispida* (Strandberg et al., 2008); and for cetaceans including dusky dolphins, *Lagenorhynchus obscurus* (Grahl-Nielsen et al., 2010); Atlantic spotted dolphins, *Stenella frontalis* (Quérouil et al., 2013); white whales,

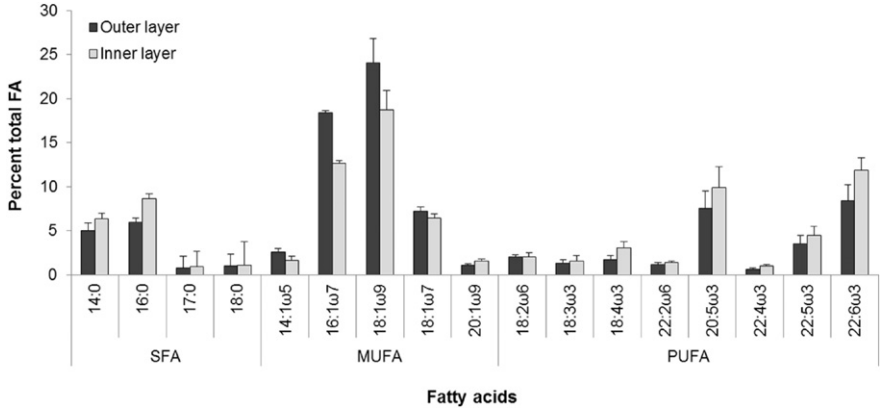

Fig. 1. Proportion of fatty acids (FAs) in the blubber of crabeater seals. Per cent total of 17 FAs in the inner and outer blubber layers of 20 crabeater seals.

Delphinapterus leucas (Dahl et al., 2000); fin whales, *Balaenoptera physalus* (Ruchonnet et al., 2006). These four FAs have been identified as endogenous, indicating that they can be readily synthesised by the animal (Herman et al., 2005; Iverson, 1993); therefore they should not be influenced by the type of prey consumed by the predator. In fact, most of these FAs are not only ubiquitous to marine mammals but are also abundant in terrestrial mammals. For example, the FA 18:1ω9 comprises about 57% of the fat of the 13-lined ground squirrel, *Ictidomys tridecemlineatus* (Price et al., 2013), and 43% in the brown bear, *Ursus arctos* (Käkëla and Hyvärinen, 1996).

The FA 16:0 is generally more abundant in terrestrial than in marine mammals, with values higher than 20% in domestic pigs, *Sus scrofa domesticus* (Apple et al., 2009; Mackay et al., 2013); grey wolfs, *Canis lupus*; brown bears; and raccoon dogs, *Nyctereutes procyonoides* (Käkëla and Hyvärinen, 1996); whereas the values of 16:0 in marine mammals fluctuate between ~4% in bottlenose whales, *Hyperoodon ampullatus* (Hooker et al., 2001), and ~15% in New Zealand sea lions, *Phocarctos hookeri* (Lambert et al., 2013) with the exception of manatees, *Trichechus manatus* (Ames et al., 2002), which have abundance of 16:0 (~26%) compared to their marine counterparts.

Another abundant FA in crabeater seals is 14:0. This is a partially endogenous FA, which can be biosynthesised by the animal but can also have a dietary origin (Iverson, 1993). The value of crabeater seals is not different from values found in other marine mammals (e.g. Arriola et al., 2013; Guerrero et al., 2016; Thiemann et al., 2008)

The other two FAs found in high proportions in crabeater seals are 22:6ω3 (DHA) and 20:5ω3 (EPA). These FAs originate from the diet (Iverson, 1993; Raclot et al., 1998) and are particularly abundant in krill and other crustaceans (Phleger et al., 2002). Similar high values of DHA and EPA have been found in arctic seals, including ringed seals (Grahl-Nielsen et al., 2005; Käkëla et al., 1993; Strandberg et al., 2008), ribbon seals, *Phoca fasciata* (West et al., 1979); and bearded seals, *Erignathus barbatus* (West et al., 1979). In Antarctic seals, on the other hand, DHA and EPA are not very abundant. Weddell and southern elephant seals have ~5% of DHA and ~3% of EPA (Best et al., 2003; Wheatley et al., 2007) whereas leopard seals have ~6% and ~5%, respectively, which may be due to leopard seals preying on krill (Guerrero et al., 2016). Humpback whales, *Megaptera novaeangliae*, who are also krill consumers, have reported ~6% of DHA and ~8% of EPA in their outer layer (Waugh et al., 2012); because of their dietary origin, these values are expected to be higher in the inner layer. Crabeater seals are krill specialists (Hückstädt et al., 2012); therefore their values of ~10% of DHA and ~12% of EPA in the inner blubber layer would indicate the dietary influence in their FA composition.

Contrarily, high levels of 20:1ω9 and 22:1ω11 tend to be characteristic of many teleost fish (Iverson et al., 1997); therefore they are expected to be more abundant in fish consumers. Crabeater seals have ~1.5% of 20:1ω9, which is low compared to southern elephant seals (Best et al., 2003); harp seals (Tucker et al., 2009); and hooded seals, *Cystophora cristata* (Tucker et al., 2009); with ~15% of 20: 1ω9. The other FA, 22:1ω11, was only present in trace amounts in crabeater seals. This could indicate that crabeater seals are not incorporating large proportions of fish in their diet; however, a quantitative analysis, including potential prey FA composition, would be necessary to estimate the actual contribution of each prey item to the crabeater seal's diet.

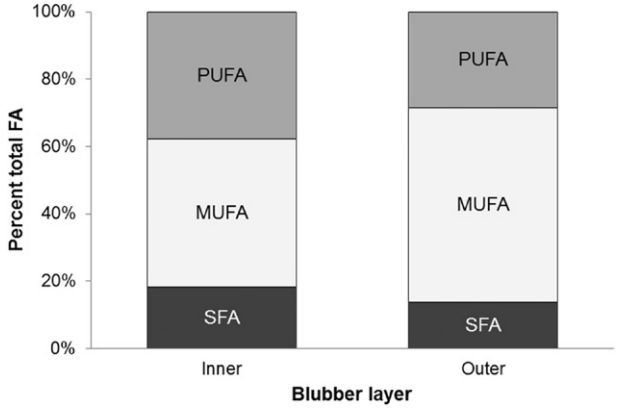

Fig. 2. Fatty acid types in inner and outer blubber layers. Mean proportion of polyunsaturated (PUFA), monounsaturated (MUFA) and saturated (SFA) fatty acids for crabeater seals, *L. carcinophaga* (n = 20).

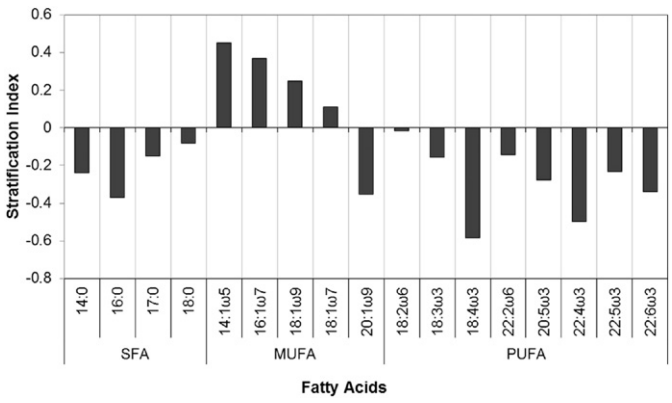

Fig. 3. Stratification index for fatty acids in inner and outer blubber layers of crabeater seals, *L. carcinophaga* (n = 20). Positive values indicate a greater proportion of the fatty acid in the outer layer and negative values indicate a greater abundance in the inner layer.

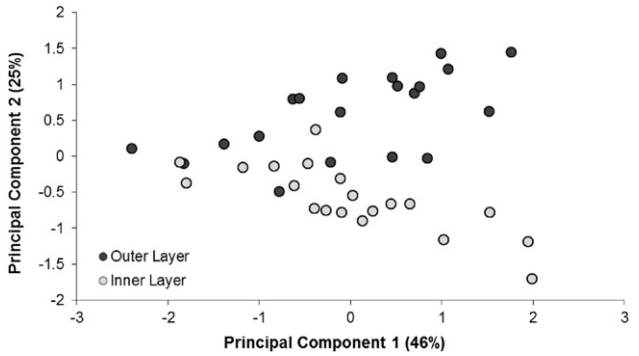

Fig. 4. Stratification of fatty acids across crabeater seal blubber. Principal component (PC) plot for fatty acids in the inner and outer blubber layers of crabeater seals, *L. carcinophaga* (n = 20).

4.2. Stratification of fatty acids: the inner layer and implications for dietary studies

Stratification implies that FAs are being deposited/mobilised differentially through the depth of the blubber core (Koopman, 2007). The identification of stratification in marine mammal blubber is important for dietary studies. When outer and inner blubber layers are substantially different, the use of the inner layer is recommended for dietary prediction (Grahl-Nielsen et al., 2005; Koopman, 2007; Olsen and Grahl-Nielsen, 2003).

Early studies in the biochemical composition of whale blubber suggested that the inner layer was more metabolically active and had a role related to the dietary process (Ackman et al., 1965; Lockyer et al., 1984). Similarly, undernourished harbour porpoises, *Phocoena phocoena*, and bottlenose dolphins have been found to reduce the number and size of adipocytes (lipid cells) only in the inner blubber layer whereas the outer layer remains stable (Koopman et al., 2002; Struntz et al., 2004). This indicates that the dietary process of depletion and accumulation of lipids occurs in the innermost section of the blubber layer (Struntz et al., 2004)

The higher proportion of PUFAs found in the inner layer of crabeater seals supports this idea (Fig. 2). These FAs originate in lower trophic levels and they move up the food web as they are consumed by consecutively higher level predators (Hoberecht, 2006). Because mammals do not have the ability to synthesise PUFAs (Iverson, 1993), the higher presence of these FAs in the inner layer suggests that the deposition/mobilisation of dietary FAs takes place here. Furthermore, when inner and outer layers have been used for dietary studies, the inner layer is closest to the composition of the potential diet (Andersen et al., 2004; Guerrero et al., 2016; Olsen and Grahl-Nielsen, 2003).

The higher proportion of SFAs in the inner layer also supports the idea of its dietary role. These FAs offer more chemical energy per unit mass (Maillet and Weber, 2006); therefore they can be used as an energy resource when needed.

Although the inner blubber layer reflects the diet of the predator better than the outer layer, the use of blubber FAs for diet inference is still under debate (Grahl-Nielsen et al., 2011; Grahl-Nielsen and Mjaavatten, 1991; Iverson et al., 2004). Most of the dietary studies have found that the inner layer does not exactly match the FAs of the prey (Best et al., 2003; Grahl-Nielsen et al., 2011); very few studies have found close resemblance between predator and prey FAs (e.g. Hooker et al., 2001).

As a specialist top predator (Hückstädt et al., 2012), the crabeater seal is a good candidate to test whether the use of FA analysis is valid for dietary predictions. This study provides the FA composition of inner and outer blubber layers of this marine mammal, which can be used for future trophic studies. In order to reduce the effect of stratification and metabolic modification of FAs, the use of the inner blubber layer and dietary FAs is recommended. Thus, a more accurate dietary prediction could be obtained.

4.3. Stratification of fatty acids: the role of the outer layer

The pattern of stratification seen in crabeater seals was similar to that found in most marine mammals (e.g. Arnould et al., 2005; Grahl-Nielsen et al., 2005; Lockyer et al., 1984; Meier et al., 2016), with MUFAs more abundant in the outer compared to the inner layer, and SFAs with the opposite tendency (Fig. 2). This suggests that the drivers behind the FA stratification in marine mammals are the same in all species (Olsen and Grahl-Nielsen, 2003). One explanation to the higher level of desaturation (higher presence of MUFAs) in the outer layer, is that these FAs are not readily mobilised by the animal; therefore they accumulate in this blubber layer (Koopman et al., 1996). Another explanation is the potentially thermoregulatory role of the outer layer (Grahl-Nielsen et al., 2005). A higher proportion of MUFAs in the outer blubber layer would lower its solidifying point, making the tissues less rigid in cold temperatures (Irving and Hart, 1957). Since most MUFAs and SFAs are endogenous FAs (Iverson, 1993), animals have the ability to synthesise higher amounts of MUFAs, modifying their saturated counterparts (SFAs). For example, the SFA C16:0 may not be abundant in the outer layer, since it has been turned into the MUFA 16:1ω7, because the latter withstands lower temperatures without solidifying. Thus, the inner layer has higher proportions of SFAs, which are solid at room temperature (Christie, 1982), but in the warm inner layer this is not a problem. However, in the cold outer layer, higher amounts of SFAs would make the tissue very rigid, therefore the transformation of SFAs into MUFAs, or desaturation of FAs, is necessary.

While this pattern of stratification appears to be universal, the degree of stratification varies among marine mammals. Koopman (2007) found increased stratification in odontocetes inhabiting colder waters, compared to species from warmer habitats, suggesting that the thermal regime is a significant factor shaping the structure of the blubber. Additionally, studies on the thermal properties of the blubber of three cetacean species have reported that the outer layer have lower thermal flux values compared to the inner layer (Bagge et al., 2012; Dunkin et al., 2005). Therefore, Bagge et al. (2012) suggests that the function of blubber as insulator may depend on its stratification and the different heat-storage capabilities of outer and inner layers. These findings suggest that crabeater seals, which are inhabitants of an extremely cold environment, should have a high degree of FA stratification compared to phocids from tempered regions.

Koopman (2007) states that the blubber of odontocetes may be more stratified than that of most other marine mammals. For example, harbour porpoises have a stratification index of 0.79 (calculated from Koopman et al., 1996) and dusky dolphins of 0.81 (calculated from Grahl-Nielsen et al., 2010). On the other hand, otariids possess a blubber layer with the lowest values of stratification, including cape fur seals with 0.10 and New Zealand sea lions with 0.14 (Arnould et al., 2005; Lambert et al., 2013). In phocids, the difference between outer and inner layers is more pronounced than in otariids (Lambert et al., 2013), which also supports the idea of the thermoregulatory role of a highly stratified blubber, since otariids in general inhabit warmer environments than phocids.

However, this does not explain the high stratification values of odontocetes, as they also inhabit temperate waters. The variation in stratification is complex and likely related to other factors such as taxonomy, blubber thickness and body size (Koopman, 2007; Lambert et al., 2013).

Compared to their Antarctic counterparts, the overall FA stratification index of crabeater seals (0.27) is lower than that of leopard seals (0.44) from the same region in western Antarctica (Guerrero et al., 2016), but higher than that of Weddell seals (0.23) from eastern Antarctica (calculated from Wheatley et al., 2007). Although these three species inhabit similar climatic regions and therefore their

stratification values are expected to be similar, the stratification index may be affected by other factors. Weddell seals in the mentioned study were sampled post-partum (Wheatley et al., 2007), which means they were probably using the lipids stored in the inner layer, which could account for the low values of stratification. In the case of crabeater seals, it is very likely that these animals had not completely recovered the inner layer utilised during the spring breeding season (Southwell et al., 2003); as samples were taken in mid-summer and they reach a maximum thickness towards the end of summer or beginning of autumn (McDonald et al., 2008); therefore the differences between inner and outer layer could have been underestimated.

Additionally, if the synthesis of MUFAs in the outer layer is a consequence of low temperatures, animals with thinner blubber layers could be expected to have more similar inner and outer layers, since cold temperatures could affect both layers. However, in this study the stratification of blubber FAs did not show any association with blubber thickness, which coincides with what was found in odontocetes by Koopman (2007). The density of fur could also impact the degree of the stratification, since this would affect the temperature at which the outer layer is maintained. Consequently, although crabeater, Weddell and leopard seals share a similar environmental temperature, it is necessary to consider other factors in order to understand their differences in the degree of blubber FA stratification.

Stratification of FAs has been explained by the influence of several roles played by this tissue (Lambert et al., 2013). There may be complex factors structuring the overall composition of the blubber and they can function differently in every species or even at an individual level. However, this aspect of the blubber composition should not be overlooked as new data could contribute to the understanding of the drivers of FA metabolism at a bigger scale.

5. Conclusions

This study provides the first description of FA composition of the blubber of crabeater seals. The most abundant FAs found here are also present in great amounts in other marine mammals. However, high proportions of DHA and EPA, are not ubiquitous in other mammals and suggest the influence of diet in the blubber FAs of crabeater seals.

Inner and outer layers were significantly different in crabeater seals, which confirms the existence of a vertical variation, or stratification, of blubber FAs. The stratification pattern was similar to that found in most marine mammals, with MUFAs more abundant in the outer layer and SFAs in greater proportions in the inner layer. This stratification suggests that inner and outer blubber layers have different functions. The higher proportion of exclusively dietary FAs in the inner layer suggests that this stratus plays a diet-related role, whereas the higher amount of MUFAs in the outer layer suggests a more functional and structural role.

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Appendix 3

Supplementary material for Chapter 4.

Appendix 2. Desaturation index (Δ9-DI) and hair density for the 48 mammalian species used in the study. Blubber section indicates whether samples analysed correspond to the whole core of blubber or just the section closer to the skin (outer layer). Data sources are indicated for fatty acid and hair density data.

Species name	Environment	Latitude	n	Blubber section	Δ9-DI	Hairs/ mm^2	Fatty acid data source	Hair data source
Rangifer tarandus	terrestrial	78	23	whole core	0.63	-	Pond et al 1993	-
Canis lupus	terrestrial	65.11	2	outer	1.02	-	Kakela & Hyvarinen 1996a	-
Nyctereutes procyonoides	terrestrial	61.38	11	outer	1.26	-	Kakela & Hyvarinen 1996a	-
Sus scrofa	terrestrial	38.63	72	outer	1.29	-	Apple et al 2008	-
Taxidea taxus	terrestrial	41.9	158	whole core	1.30	-	Harlow & Varnell 1980	-
Ursus arctos	terrestrial	65.11	4	outer	1.53	-	Kakela & Hyvarinen 1996a	-
Vulpes lagopus	terrestrial	71.38	4	whole core	2.13	-	Shultz & Ferguson 1973	-
Ictidomys tridecemlineatus	terrestrial	49.5	6	outer	3.41	-	Price et al 2013	-
Castor canadensis	semi-aquatic	65.11	4	whole core	0.68	373	Kakela & Hyvarinen 1996b	Fish et al., 2002
Castor fiber	semi-aquatic	65.11	4	whole core	0.94	324	Kakela & Hyvarinen 1996b	Wilfried et al., 2011
Ondatra zibethicus	semi-aquatic	65.11	7	whole core	0.97	396	Kakela & Hyvarinen 1996b	Fish et al., 2002
Lutra lutra	semi-aquatic	65.11	5	outer	1.52	699	Kakela & Hyvarinen 1996a	Wilfried et al., 2011
Phocarctos hookeri	semi-aquatic	50.76	16	outer	1.71	22	Lambert et al 2013	Scheffer, 1964
Cystophora cristata	semi-aquatic	54.04	32	whole core	1.89	14	Thiemann et al 2008	Scheffer, 1964
Pagophilus groenlandicus	semi-aquatic	54.04	239	whole core	1.92	17	Thiemann et al 2008	Scheffer, 1964
Eumetopias jubatus	semi-aquatic	61.22	96	whole core	2.13	12	Beck et al 2007	Scheffer, 1964
Neophoca cinerea	semi-aquatic	33.9	2	outer	2.29	-	This study	-
Mirounga angustirostris	semi-aquatic	37.11	20	whole core	2.32	4	Noren et al 2013	Scheffer, 1964
Arctocephalus pusillus	semi-aquatic	31.6	2	outer	2.35	-	Arnould et al 2005	-
Arctocephalus tropicalis	semi-aquatic	33.9	1	outer	1.62	418	This study	Liwanag et al., 2012
Arctocephalus forsteri	semi-aquatic	33.9	1	outer	2.67	336	This study	Scheffer, 1964
Halichoerus grypus	semi-aquatic	60.12	3	outer	2.80	22	Kakela et al 1993	Scheffer, 1964
Erignathus barbatus	semi-aquatic	54.04	80	whole core	2.96	28	Thiemann et al 2008	Scheffer, 1964
Histriophoca fasciata	semi-aquatic	65.87	2	whole core	3.53	15	West et al 1979	Scheffer, 1964
Mirounga leonina	semi-aquatic	54.83	11	outer	3.85	-	Best et al 2003	-
Leptonychotes weddellii	semi-aquatic	77.85	19	outer	3.92	14	Wheatley et al 2007	Scheffer, 1964
Ursus maritimus	semi-aquatic	59.51	20	outer	3.98	29	Thiemann et al 2005	Wilfried et al., 2011
Lobodon carcinophaga	semi-aquatic	64.15	20	outer	4.39	28	Guerrero & Rogers (in press)	Scheffer, 1964
Hydrurga leptonyx	semi-aquatic	64.15	24	outer	4.51	20	Guerrero et al. 2016	Scheffer, 1964
Odobenus rosmarus	semi-aquatic	79.79	14	outer	4.75	2	Skoglund et al 2010	Scheffer, 1964
Pusa sibirica	semi-aquatic	53.5	30	outer	5.52	-	Grahl-Nielsen et al 2005	-
Pusa hispida	semi-aquatic	78.91	25	outer	7.02	200	Strandberg et al 2008	Scheffer, 1964
Phoca vitulina	semi-aquatic	78.33	5	outer	7.06	216	Andersen et al 2004	Scheffer, 1964
Trichechus manatus	fully-aquatic	28.4	17	outer	1.04	-	Ames et al 2002	-
Balaenoptera physalus	fully-aquatic	34.7	1	outer	2.08	0	Ruchonnet et al 2006	-
Phocoena phocoena	fully-aquatic	43.21	19	outer	2.29	0	Koopman et al 1996	-
Megaptera novaeangliae	fully-aquatic	27.43	17	outer	2.45	0	Waugh et al 2012	-
Balaena mysticetus	fully-aquatic	70.6	18	outer	2.62	0	Budge et al 2008	-
Balaenoptera acutorostrata	fully-aquatic	65.72	22	outer	3.17	0	Olsen & Grahl-Nielsen 2003	-
Delphinus delphis	fully-aquatic	35.27	12	outer	3.41	0	Quérouil et al 2013	-
Monodon monoceros	fully-aquatic	72	20	whole core	3.74	0	Thiemann et al 2008	-
Caperea marginata	fully-aquatic	33.9	2	outer	1.20	0	This study	-
Physeter macrocephalus	fully-aquatic	37.74	15	outer	3.86	0	Walton et al 2008	-
Delphinapterus leucas	fully-aquatic	79.05	7	whole core	3.91	0	Dahl et al 2000	-
Stenella frontalis	fully-aquatic	35.27	10	outer	3.94	0	Quérouil et al 2013	-
Grampus griseus	fully-aquatic	33.9	1	outer	1.58	0	This study	-
Lagenorhynchus obscurus	fully-aquatic	17.4	5	outer	3.99	0	Grahl-Nielsen et al 2010	-
Hyperoodon ampullatus	fully-aquatic	48.15	3	outer	4.87	0	Hooker et al 2001	-

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