Fatty acid variation in beluga (*Delphinapterus leucas*) blubber: implications for estimating diet using fatty acids

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This is to certify that I have examined this copy of a doctoral dissertation by

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Abstract

Fatty acid variation in beluga (*Delphinapterus leucas*) blubber:
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Diet is one of the most important aspects of the ecology of any species, yet remains difficult to estimate for many marine mammals. Quantitative fatty acid signature analysis (QFASA), a recently developed technique to estimate predator diets by comparing the fatty acid (FA) composition of predator tissues with that of potential prey items, is well-suited to estimating marine mammal diet as most possess a large and relatively easily-accessed blubber layer. However, prior to making diet inferences using FA, it is imperative to examine FA variability within the blubber layer and potential impacts of this variability on QFASA diet estimates. Fatty acid composition of beluga (Delphinapterus leucas) blubber from eastern Chukchi Sea belugas was examined. Composition did not vary across the dorsal portion of the thorax, but FA were stratified with depth in the blubber, and the degree of stratification increased with age. Isovaleric acid, a short-chain FA that is not derived from diet, was investigated separately. Isovaleric acid was found in minute amounts in the liver and much larger amounts in the blubber and melon, supporting a hypothesis of local biosynthesis in the blubber and melon. Isovaleric acid was also

stratified with depth in the blubber, and the degree of stratification increased with age. Finally, the impact of several sources of FA variation was explored on QFASA diet estimates. Diet estimates were insensitive to laboratory technique used to process fat samples (methyl esters vs. butyl esters) and the sample collection site on beluga thorax. However, blubber sampling depth affected diet estimates in terms of both prey species identified and proportions of the diet they represented. This study illustrates the great promise in using QFASA to estimate diet in belugas and other cetacean species, and can be used to guide sample collection protocols. While the production of accurate diet estimates remains contingent upon satisfying QFASA model requirements, this study clearly shows that collecting cetacean blubber and potential prey samples is already a worthwhile endeavor, and that baseline datasets can now be developed for detection of future changes.

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CHAPTER ONE

INTRODUCTION

Diet is one of the most important aspects of the ecology of any species, yet remains difficult to estimate for many marine mammals, particularly cetaceans. With few exceptions, marine mammal foraging behaviour is nearly impossible to observe in the wild. Traditional dietary assessment techniques can be difficult to apply to marine mammals and are subject to many well-known biases. Analyses of scats and stomach contents are primarily limited to animals that are dead, captive, or haul out on land, and are biased towards prey species with hard body parts that are not easily digested (da Silva & Neilson 1985, Pascoe 1986, Pierce et al. 1991). While work has been done to develop correction factors that account for otoliths and cephalopod beaks that have not been completely digested (Bowen 2000, Orr & Harvey 2001), these correction factors are only applied to prey species present in the stomach or fecal sample. Thus, prey species without representative hard parts are not accounted for in the analyses and are therefore presumed to be absent from the diet. Additionally, fecal and stomach samples only provide information on recently consumed meals (Jobling & Breiby 1986, Orr & Harvey 2001), potentially limiting the identification of prey species to a localized area where the sample or animal was collected.

Fortunately, chemical methods now exist that allow for the indirect estimation of marine mammal diets. The biochemical composition of many body

tissues is greatly influenced by the prey items ingested by an animal. Our understanding of the processes influencing the chemical makeup of various tissues is such that it is now possible to correlate certain chemical signals with various aspects of foraging ecology and behaviour. In addition, technology has advanced to the point where it is now feasible to measure these chemical signals in relatively large numbers of individuals, allowing us to sample animals at ecologically relevant scales and to make inferences about diet with some degree of statistical confidence.

Analysis of stable isotopes and contaminants are two biochemical methods that are minimally invasive and can be carried out on free-ranging animals. However, these techniques generally cannot estimate species composition of diet, instead providing qualitative information on geographic location of prey species consumed (e.g., Hobson 1999, Krahn et al. 2007) and trophic level of the consumer (e.g., Rau et al. 1992, Lesage et al. 2001, Das et al. 2003). Mixing models have been developed to identify and estimate proportions of individual prey species based on stable isotope data, but these are of limited value when the number of potential prey species exceeds the number, typically only carbon and nitrogen, of stable isotopes measured (Phillips 2001). More promising biochemical techniques involve the use of fatty acids (FA) in fat storage tissues, milk, and blood (Iverson 2009a).

Estimating diet using blubber fatty acid data

Quantitative FA signature analysis (QFASA) is a recently developed technique used to estimate predator diets by comparing the FA composition of

predator tissues with potential prey items, after accounting for predator lipid metabolism (Iverson et al. 2004). QFASA has been used to estimate diets in numerous predators (e.g., Iverson et al. 2004, Iverson et al. 2006, Beck et al. 2007, Iverson et al. 2007, Nordstrom et al. 2008, Tucker et al. 2009), and is the most powerful of the chemical methods used to estimate diet. The advantages of QFASA include the identification of prey items without hard parts, estimation of proportions of prey items (by species and potentially by life stage/size), estimation of diet over longer time scales (thereby also increasing the spatial scale over which diet is estimated), and non-lethal sampling that may be used on free-ranging animals (e.g., Iverson et al. 2004, Beck et al. 2007, Tucker et al. 2009).

QFASA is especially well-suited to estimating diet in marine mammals as most possess a large, relatively easily-accessed fat store in the form of their blubber layer. Blubber is a dense and vascularized layer of specialized adipose tissue that lies beneath the skin in pinnipeds and cetaceans. In addition to being the primary and most important site of fat and energy storage in most marine mammals, blubber also has a number of other functions: it provides insulation for mammals living in often cold marine environments, and may aid in efficient locomotion, functioning as a buoyancy adjuster, a body streamliner and biomechanical spring (Iverson 2009a). Blubber is a biochemically dynamic tissue, filling with fat during periods of feeding and positive energy balance, and mobilizing fat during periods of fasting (Iverson 2009a).

FA are the building blocks of the majority of lipids in adipose tissue, including blubber (Figure 1.1). When lipids are digested, lipases in the predator gut hydrolyze dietary triacylglycerols (TAG), composed of three FA esterified to a glycerol molecule. The FA released are then absorbed by cells lining the intestine, with longer chain FA eventually being taken up by blubber adipocytes and re-esterified into TAG for storage (Pond 1998). As the FA elongation and desaturation capabilities of monogastric consumers such as marine mammals are limited (Cook 1985, Nelson 1992), dietary FA are largely conserved and deposited directly, or predictably, in the blubber layer, thus providing insight into nutritional state and individual feeding habits and foraging ecology that are otherwise difficult to study.

Nevertheless, blubber as a tissue is likely adapted to best suit the lifestyles, stresses, and constraints of specific groups and even individual species of marine mammals. Hence, the study of blubber can also provide unique insights into phylogenetic relationships, lipid metabolism, and environmental adaptations (Koopman 2007, Iverson 2009a). In contrast to pinnipeds, which possess a relatively homogenous blubber layer, the blubber of cetaceans tends to be biochemically stratified. In addition, in some odontocetes blubber contains relatively large concentrations of an unusual very short-chain FA, isovaleric acid (*i*-5:0), which is synthesized somewhere in the body and does not come from diet (e.g., Koopman et al. 2003, Koopman 2007). Thus, an increased understanding of non-dietary factors constraining the FA makeup of cetacean, and especially odontocete, blubber will

improve the ability to make inferences about their diets based on observed variation in FA composition.

Study animal

Belugas (*Delphinapterus leucas*) are the most abundant cetacean inhabiting seasonally ice-covered circumpolar arctic and sub-arctic waters (Hazard 1988) (Figure 1.2). These ice-adapted odontocetes have thickened skin on the dorsum, lack a dorsal fin, and undergo annual migrations often coincident with the formation and retreat of the pack ice (Burns et al. 1981, Hazard 1988). Belugas are sexually dimorphic and vary significantly in maximum and average body sizes between populations. Males reach lengths of 5 metres and weights of 1,200 kg, while females reach lengths of 4.5 metres and weights of 700 kg (Laidre 2007). Belugas in the wild are thought to live as long as 60 years (Stewart et al. 2006), with a calving interval of three years (Seaman & Burns 1981).

Research on belugas in Alaska has led to an increased understanding of stock structure, migration routes, habitat use, and population dynamics through the use of genetics, telemetry, and harvest sampling (Suydam et al. 2001, O'Corry-Crowe et al. 2002). Despite the abundance and likely importance of belugas as top predators, current knowledge of beluga diet is limited to stomach contents analysis and observations made by beluga hunters (Brooks 1955, Seaman et al. 1982, Frost & Lowry 1984, Huntington 1999). Belugas have been described as generalist predators and are thought to take prey which is most abundant and available. In Alaska,

beluga stomachs have been found to contain shrimp, octopus, and various species of cod, sculpin, flatfish, and salmon (L. Quakenbush, *unpublished data*). However, these data are subject to previously-mentioned biases, in particular that diet estimates are restricted to the brief window of time and range of locations immediately prior to the death of whales sampled during harvests. Thus, FA analysis is a potentially attractive tool for gaining insight into beluga diet, as it would provide information on diet integrated over a period of months, and samples can be collected using remote biopsy techniques on free-ranging animals.

There are at least five designated stocks of belugas in Alaska, one of which, the Cook Inlet stock, is listed as "endangered" under the U.S. Endangered Species Act (ESA). Belugas are an important subsistence resource for many communities in coastal Alaska (Seaman & Burns 1981), and there is great concern about impacts to belugas and subsistence hunting from rapid climate change.

The four unlisted stocks of belugas in Alaska are thought to share wintering grounds in the eastern Bering Sea, while differing in spring migration routes and summering areas. Not only are all the areas utilized by belugas subject to warming climate and ecosystem shifts (Grebmeier et al. 2006, IPCC 2007), the migration routes and summering areas of the eastern Chukchi and Beaufort Sea beluga stocks overlap with areas used for oil and gas exploration/development (Richard et al. 2001, Suydam 2009). Interest in the exploration and development of oil and gas reserves in the Chukchi and Beaufort seas has grown tremendously. Diet information is vital for making informed decisions about beluga conservation and management, whether

related to climate change, hunting, or oil and gas activities. Describing current beluga diet will also provide a baseline for future comparisons and impacts of climate change.

The application of FA analysis to beluga blubber has been employed by several researchers in recent years (Dahl et al. 2000, Nozeres et al. 2001, Krahn et al. 2004, Birkeland et al. 2005, Loseto et al. 2009). Whereas such analyses have allowed for some qualitative conclusions regarding beluga diet, none have used QFASA to produce quantitative estimates of potential prey items.

Scope of dissertation

The overall goals of this dissertation were to increase our knowledge and understanding of FA variation in beluga blubber and to contribute to our ability to estimate the diet of free-ranging belugas and other odontocetes using FA analysis. In Chapter 2, *Fatty acid variation in belugas,* I focused on a set of FA commonly used to estimate diet with the QFASA model. I first examined variation in FA levels with blubber depth and body site. One of the aforementioned benefits of using FA to make inferences about marine mammal diet is that this technique may be applied to free-ranging animals, with samples potentially collected via remote biopsy (Hooker et al. 2001, Hoberecht et al. 2006). However, prior to making inferences about diet using samples possibly collected from a range of body sites and blubber depths, it is imperative to examine the extent to which these factors might influence FA composition. I then examined the effects of both age and sex on the degree of FA

stratification in blubber. FA stratification has been observed to increase with sexual maturity in a number of odontocetes (Koopman et al. 1996, Smith & Worthy 2006, Koopman 2007). If the degree to which FA composition differs between inner and outer blubber layers is positively correlated with age, then it follows that collecting the innermost blubber layer to estimate diet using QFASA would be even more important in older animals.

In Chapter 3, *Isovaleric acid: evidence for local biosynthesis and the influence of age and sex,* I quantified levels of *i*-5:0 in blubber, melon, liver, and heart to investigate the idea that this FA may be biosynthesized within the blubber itself. Isovaleric acid is believed to be toxic to animals; its accumulation in the bloodstream can lead to vomiting, seizure, coma, and death in humans (Tanaka et al. 1966, Vockley & Ensenauer 2006). Thus, I hypothesized that it would be unlikely to be synthesized in the liver, necessitating transport to the blubber via the bloodstream, and exposing these animals to similar toxic effects. In Chapter 3, I also examined the effect of age and sex on both the level of *i*-5:0 and its' stratification in the blubber layer.

Finally, in Chapter 4, *The effect of non-dietary sources of fatty acid variation on quantitative fatty acid signature analysis diet estimates in an arctic odontocete,* I investigated the effects of FA variation due to layer, body location and transesterification technique used to prepare fat samples in the lab on diet estimates. While it is informative to explore such FA differences statistically, as was done in

Chapter 2, it is ecologically more meaningful to examine how these differences are manifested in terms of estimated predator diets using QFASA.

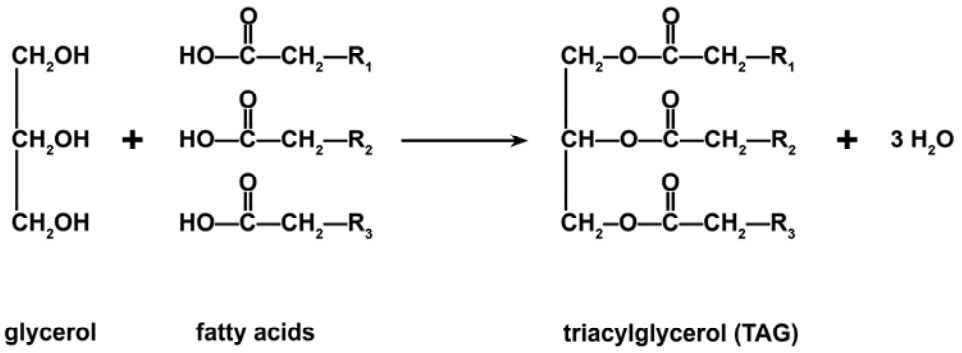

Figure 1.1. Fatty acids (FA) are the building blocks of most blubber lipids. FA are stored in blubber adipocytes as triacylglycerols (TAG), which are formed by the esterification of three FA to one glycerol molecule.

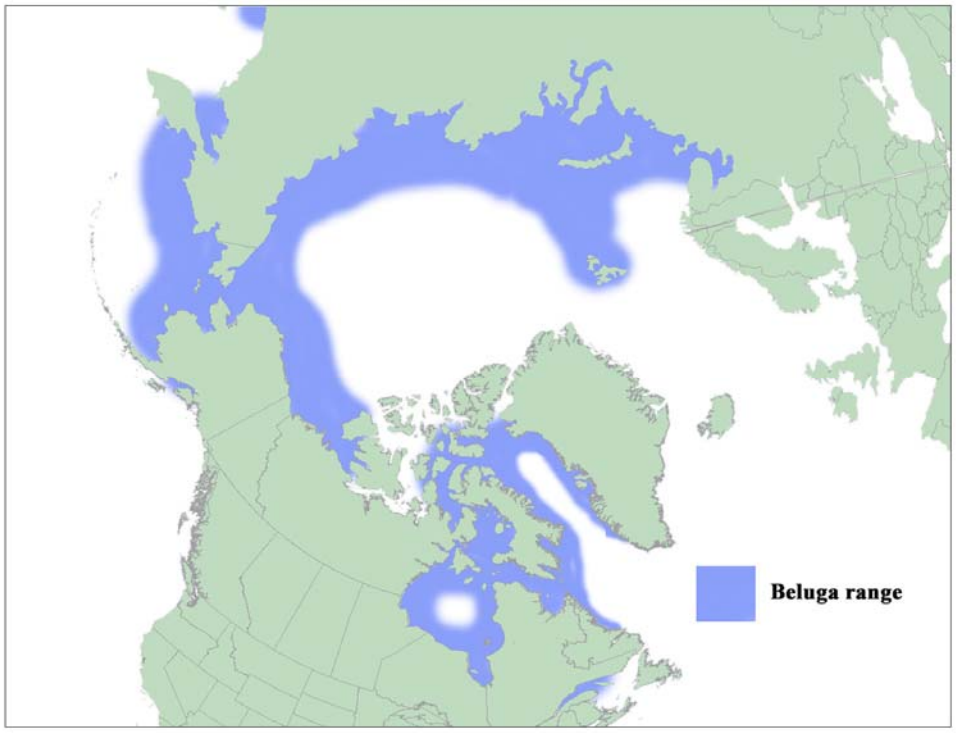

Figure 1.2. Worldwide distribution of belugas. Reprinted with permission from Laidre (2007).

CHAPTER TWO

FATTY ACID VARIATION IN BELUGAS

INTRODUCTION

The interest and value in estimating the diet of marine mammals is great, yet remains difficult for most species given that they spend much of their lives in and below the surface of the water. The diet of arctic cetaceans is particularly challenging to determine as they range across vast distances in remote and inhospitable polar regions. Traditional diet techniques, such as stomach contents and scat analysis, are primarily limited to non-cetacean species and are subject to many well-known biases. A number of biochemical methods are now used to make inferences about marine mammal diet, the most powerful of these being fatty acid (FA) analysis.

The use of FA to make both qualitative and quantitative inferences about marine mammal diet has become increasingly common in a number of marine systems (reviewed in Budge et al. 2006, Iverson 2009b). Qualitative inferences are typically based on the examination of a suite of FA, i.e., the FA signature (Iverson 1993), with comparisons made among groups of predators or between predators and potential prey using a variety of multivariate statistical techniques. Quantitative inferences are currently only produced using the quantitative FA signature analysis (QFASA) statistical model developed by Iverson et al. (2004). In addition to samples of predator fatty tissues, QFASA requires an appropriate prey library,

ideally composed of sufficient numbers of individuals of each "prey type" to rigorously assess within- and between species variability and the reliability with which they can be differentiated by their FA signatures. When using FA to make either qualitative or quantitative inferences about diet, non-dietary sources of FA variation within the predator (i.e. due to sex, season, or blubber structure) must be investigated and controlled for.

Cetaceans, particularly odontocetes, are important to understand in this regard, as their blubber appears to be among the most structured of marine mammals (e.g., Koopman et al. 1996, Koopman et al. 2003, Koopman 2007). To date, only two studies have thoroughly examined variation in FA composition of blubber sampled across various body sites in odontocetes. Koopman et al. (1996) compared the FA composition at four body sites in the harbour porpoise (*Phocoena phocoena*), while Samuel and Worthy (2004) examined FA composition at nine sites in bottlenose dolphins (*Tursiops truncatus*). In both studies, FA composition was stratified with depth, but found to be relatively uniform with location across the thorax. Additionally, Krahn et al. (2004) determined FA composition at various sites across the dorsal and lateral portions of the thorax in two killer whales (*Orcinus orca*), and also found greater variation among depth layers than between body sites. The first whale was sampled at two body sites, and the second whale was sampled at five sites (three of the five sampling sites were on the "saddle patch").

Even less is known about variation in FA composition of blubber sampled at different body sites in mysticetes. Ruchonnet et al. (2006) sampled a single

Mediterranean fin whale (*Balaenoptera physalus*) at three sites (dorsal, lateral, ventral), and found more variation due to site than blubber depth (blubber was divided into three depth layers). Budge et al. (2008) sampled four bowhead whales (*Balaena mysticetus*) at both a dorsal and ventral position, and found no significant differences in FA composition between sites in the innermost layer (blubber was divided into five layers).

In contrast to the paucity of information on FA variation across body locations, stratification with depth in cetacean blubber is well documented (Table 2.1). Blubber has been divided into a variety of layers based on a number of criteria depending on the study. Most often, blubber has been divided into two to three layers of equal thickness (e.g., Hooker et al. 2001, Smith & Worthy 2006, Koopman 2007). However, Lockyer et al. (1984) divided fin whale blubber into three layers of unequal thickness on the basis of macroscopic differences, while Krahn et al. (2004) divided the killer whale (*Orcinus orca*) blubber into layers 2 cm thick as measured inwards from the epidermis.

Regardless of the number of layers examined, the same general patterns of FA variation are observed. Monounsaturated FA (MUFA) less than 18 carbons in length are consistently found at higher levels in outer relative to inner blubber layers. In contrast, MUFA greater than 18 carbons in length, non-branched saturated FA (SFA), and many polyunsaturated FA (PUFA) are present at higher levels in the innermost versus outermost blubber (e.g., Koopman et al. 1996, Samuel & Worthy 2004, Smith & Worthy 2006).

It has long been proposed that the innermost blubber is more metabolically active than outermost blubber. Ackman et al. (1975) observed greater heterogeneity in inner versus outer blubber iodine values, which are positively correlated with the degree of unsaturation in fat, in both fin and sei (*B. borealis*) whales. The authors concluded that this represented the deposition of surplus dietary FA during periods of fattening. Aguilar and Borrell (1990) similarly concluded that the inner blubber played an active role in the deposition and mobilization of lipids in fin whales after examining variation in lipid content with blubber depth. The lipid content of outer blubber was relatively stable compared to the increased variability in the inner blubber, which was correlated with reproductive state. Increased metabolic activity in the inner blubber layer has also been proposed on the basis of FA composition. Research has shown that FA of dietary origin appear to be preferentially deposited in the inner blubber layer of odontocetes (Koopman et al. 1996, Koopman et al. 2002, Koopman 2007).

Further support for the idea that the outer blubber is less metabolically active comes from findings that the biochemical stratification in blubber consistently increases with age in odontocetes, with apparent sequestering of biosynthesized components in the outer layer. Koopman et al. (1996) found that the blubber of harbour porpoise calves and yearlings was significantly less stratified than that of porpoises older than four years of age. Using body size as a proxy for age, Koopman (2007) found that stratification increased significantly with body size in common dolphins (*Delphinus delphis*), Risso's dolphins (*Grampus griseus*), pygmy sperm

whales (*Kogia breviceps*) and bottlenose dolphins. Finally, Smith and Worthy (2006) found that the blubber of "short" body length sexually immature male short-beaked common dolphins was less stratified than that of sexually mature and "long" sexually immature dolphins.

The FA composition of beluga (*Delphinapterus leucas*) blubber has been investigated by a number of researchers, however none have examined the potential effects of either layer or body site in a comprehensive manner. Dahl et al. (2000) compared FA data from full-depth blubber cores of belugas ($n = 7$) with potential prey items using principal components analysis (PCA). Birkeland et al. (2005) found few FA present in significantly different quantities in inner versus outer blubber ($n = 4$), and hypothesized that the poorly pronounced level of stratification observed was possibly due to the "shielding" effect of the overlying "cork" layer. Krahn et al. (2004) examined the FA composition of beluga blubber divided into two ($n = 2$), three ($n = 1$) and four ($n = 2$) layers, where the number of layers was dictated by the thickness of the full-depth blubber sample collected. Their general conclusions were that FA stratification did exist within the blubber, and that lower molecular weight FA were present in higher proportions in the outer vs. the inner blubber. Stratification in beluga blubber has also been documented by Koopman (2007) ($n = 26$). Koopman (2007) further examined the degree to which stratification was related to body size in a number of odontocetes, and found no significant relationship for belugas.

Blubber FA have been used to make inferences about diet of belugas in the eastern Beaufort Sea (Loseto et al. 2008, Loseto et al. 2009). Loseto et al. found that beluga diet was a function of body length (2008), and determined that the summer diet of belugas was predominated by Arctic cod (*Boreaogadus saida*) (2009). In these studies, only the FA composition of the inner blubber was examined, with the work of Koopman et al. (2002) on harbour porpoise as the justification. Finally, Nozeres et al. (2001) compared the FA composition of the inner blubber of Saint Lawrence belugas with four species of sympatric pinnipeds using PCA and hierarchical clustering analysis. Belugas were found to segregate by sex and year, and it was suggested that beluga and harbour seal (*Phoca vitulina*) diets differed on the basis of the relatively high levels of 20:1*n*-9 and 22:1*n*-11 measured in belugas. Nozeres et al. (2001) divided the beluga blubber into three equal layers (C. Nozeres, *personal communication*).

Prior to using FA data to make inferences about marine mammal diet, it is imperative to determine whether blubber FA composition varies with sampling site on the body and/or with blubber depth. This is especially important for researchers studying free-ranging belugas and other cetaceans, which may be sampled via remote biopsy dart and therefore from a range of body sites and perhaps without the collection of the innermost portion of the blubber.

Chapter Objectives

I examined variation in FA composition within the blubber of individual belugas, with respect to both blubber depth and sampling site on the thorax (the area most likely to be sampled by remote biopsy). I also investigated the effects of age and sex on FA stratification within the blubber. For these examinations, I used a set of FA commonly used to estimate diet with the QFASA model, as the variation in these FA would be most relevant to issues in diet estimation.

METHODS

Sample collection

Blubber samples were collected from 95 belugas harvested by subsistence hunters in the village of Point Lay, Alaska (Figure 2.1), in late June/early July in 2002, and in each year from 2005 to 2008. Approximately 20 animals were sampled each year. The impacts of blubber depth and sampling site on FA composition were addressed using only samples collected in 2002 (n = 20). In 2002, belugas thought to be adults based on size and skin colour (adult animals are white, whereas younger animals are grey (Brodie 1989)) were preferentially sampled in an attempt to reduce the possible effects of age on FA composition (Table 2.2). Full depth blubber cores were collected from all 20 belugas at each of six body sites, spanning the portion of the thorax most likely to be sampled via biopsy dart in free-ranging animals (Figure 2.2).

The effects of age and sex on FA stratification were addressed using samples collected in all years (Table 2.3). The majority of the animals sampled were relatively large males, despite sampling all available small and/or female animals, because of size- and sex-specific biases in the hunts (Suydam 2009). Blubber was only sampled at body site # 4 (Figure 2.2) in 2005 to 2008.

Blubber cores were collected and frozen at -20 °C within approximately 24 hours of death. Blubber cores had skin attached, and were roughly 5 cm x 5 cm in cross section, allowing samples to be trimmed of freezer-burned tissue immediately prior to lipid analysis in the lab. Straight-line body length, from the tip of the snout to the notch in the flukes, was measured to the nearest cm for all animals. Half of the lower jaws, with teeth to be used for age determination, were collected from 46 of these animals.

Belugas were sampled with the permission and cooperation of the elders and people of Point Lay, AK. Samples were collected under the U.S. Marine Mammal Protection Act (MMPA) Permit No. 782-1694-02 to the National Marine Mammal Laboratory, Alaska Fisheries Science Center, National Oceanic and Atmospheric Administration.

Lipid analysis

Blubber was divided into three equal depth layers: inner (adjacent to muscle), middle and outer (adjacent to skin) (Figure 2.3). Lipids were extracted using the Folch procedure (Folch et al. 1957), modified as described by Budge et al. (2006).

Briefly, lipids were mashed from 0.5 g pieces of blubber into 2:1 chloroform: methanol with 0.01 % BHT (v/v/w) added as an anti-oxidant. This mixture was washed with 0.7 % NaCl and centrifuged to separate the lipid-containing chloroform layer. The chloroform layer was then dried with anhydrous sodium sulfate and evaporated under nitrogen to yield the lipid extract.

Fatty acid butyl esters (FABE) were prepared instead of the more commonly used fatty acid methyl esters (FAME) to allow for the quantification of short-chain FA such as isovaleric acid (*i*-5:0) known to be present in beluga blubber (Koopman 2007). FABE were prepared by heating extracted lipid in hexane with 10 % boron trifluoride (BF_3) in butanol at 100 °C for one hour (Budge et al. 2006). Lastly, FABE in hexane were dried with anhydrous sodium sulfate.

FABE were analyzed in duplicate using temperature programmed gas-liquid chromatography. Separations were carried out on an Agilent HP 6890 gas chromatograph (GC) fitted with a 30 m × 0.25 mm ID flexible fused silica column coated with (50%-Cyanopropyl)-methylpolysiloxane (0.25 μm film thickness; DB-23; Agilent Technologies, USA). Hydrogen was used as the carrier gas, and the gas line was equipped with oxygen and hydrocarbon scrubbers. Oven programming from Budge et al. (2006) was used. The initial oven temperature of 65 °C was held for 2 min, ramped to 165 °C at 20 °C/min, held for 0.4 min, ramped to 215 °C at 2 °C/min, held for 3 min, ramped to 235 °C at 5 °C/min, and then ramped to 240 °C at 25 °C and held for 0.5 min, producing a total runtime of approximately 40 minutes. The GC was linked to a flame-ionization detector (FID). The GC-FID was

controlled by, and peaks identified and integrated using, ChemStation software (Rev A.10.02, Agilent Technologies, USA). FA were identified by comparison with commercially prepared and known reference mixtures. Individual FA were converted to mass percent of total FA identified by applying theoretical response factors (TRF) relative to stearic acid (18:0) (Ackman & Sipos 1964, Ulberth et al. 1999). FA are named using the shorthand nomenclature of C:D*n*-x, where C is the number of carbon atoms, D is the number of double bonds, and *n*-x denotes the position of the first double bond relative to the terminal methyl end of the FA.

Age determination

Age was determined by counting growth layer groups (GLG) in thin sections of teeth. Jaws were boiled, and teeth were extracted and cleaned. The three largest and least-worn teeth from each beluga were mounted on a small wooden block and thinly sectioned using a Buehler IsoMet low-speed saw equipped with a diamond wafering blade. For each beluga, GLGs in at least two teeth were counted a minimum of two times each by two readers. If needed, additional teeth were counted until an agreed-upon final count for each animal was reached by both readers (Lockyer et al. 2007). Actual age was estimated for animals when the neonatal line of the tooth was visible, while minimum age was estimated for older animals when the neonatal line had been worn away. In accordance with the findings of Stewart et al. (2006), one GLG was assumed to form annually.

Data analysis

FA data were transformed using the following additive log-ratio approach advocated by Aitchison (1994) for use with compositional data:

$$x_{trans} = \ln(x_i/c_r)$$

where x_{trans} is the transformed FA, x_i is the FA expressed in mass percent of total FA identified, and c_r is the mass percent value of a reference FA. Vaccenic acid (18:1*n*7) was chosen as the reference FA, as it is commonly quantified in samples processed by most researchers currently active in this field (e.g., Koopman et al. 1996, Walton et al. 2007, Strandberg et al. 2008), and because a preliminary analysis of this dataset indicated that mass % values of 18:1*n*7 were approximately equal in all three layers.

Although 70 FA were identified in each sample, only 36 were selected for data analysis as the objective here was to examine FA variation in the context of its potential impact on diet inferences. The 36 FA were selected on the basis of their inclusion in the "extended" FA subset defined by Iverson et al. (2004) (Table 2.4). Not all FA quantified in predator samples are equally useful for informing diet inferences (summarized in Iverson et al. (2004)). The "extended" subset consists of FA that could have only come from ingested prey items, in addition to several FA that may be biosynthesized by the predator, but in quantities influenced by specific prey items ingested. Although 18:1*n*7 is a member of the extended FA subset, it was selected as the denominator in the transformation described above, and was thus excluded from the statistical analysis and examined visually instead.

Fatty acid variation with body site and blubber depth

A two-factor MANOVA using all 35 FA, followed by univariate two-factor ANOVAs and Tukey's HSD post-hoc tests ($\alpha = 0.05$) for each individual FA (SPSS for Windows, version 11.5; SPSS Inc, 2002), were used to investigate the effects of body site and blubber depth on FA composition. Individual belugas were used as blocks, and the Bonferroni method of modifying the alpha value ($\alpha = 0.05/35 = 0.0014$) was used to reduce the increased risk of Type I errors in association with multiple F-tests (Johnson & Wichern 1998).

Effects of age, body length, and sex on fatty acid stratification

FA stratification was explored in terms of the average stratification across all FA in the extended FA subset. Accordingly, the stratification index (SI) was calculated as follows:

$$SI.mean.ext = \frac{\sum_{i=1}^{35} (|FA_{inner} - FA_{outer}|)}{35}$$

for the 35 FA in the "extended" subset (note: 18:1n7 was not included in SI.mean.ext as it was used as the denominator in the data transformation described above).

An ANCOVA was used to examine the effect of "age" on SI.mean.ext, and sex was included in the model as a factor. The analysis was first performed on the full set of observations with GLG measurements ($n = 46$), and then repeated using body length as the independent variable in order to make use of the full set of

belugas sampled (n = 95). Interaction between sex and the covariate (GLGs or length) was evaluated by including an interaction term in the model for the ANCOVA. If the interaction term was found to be nonsignificant, the ANCOVA was re-run with the interaction term removed. If sex was subsequently found to be nonsignificant, a simple linear regression was done. All testing was done at α = 0.05. Normality was assessed by examining boxplots and QQ-plots prior to the ANCOVA, and residual plots afterwards. Homogeneity of variances was assessed using Levene's Test.

RESULTS

MUFA dominated all samples, with notably high levels of 16:1*n*7, 18:1*n*9, 20:1*n*11, 20:1*n*9 and 22:1*n*11. Branched-chain (e.g., *i*-5:0) and SFA less than 13 carbon atoms in length were present in higher amounts in the outer versus inner blubber layers, and were dominated by *i*-5:0. All other SFA were present in higher amounts in the inner versus outer blubber layers, and were dominated by 14:0 and 16:0. PUFA composed the smallest proportion of total FA, were typically present in higher amounts in the inner versus outer blubber layers, and were dominated by 20:5*n*3, 22:5*n*3 and 22:6*n*3 (Table 2.4).

Boxplots for individual FA showed no obvious skewness, and plots of residuals versus predicted values showed no obvious pattern, suggesting the assumptions of normality and equal variance were reasonable. A few potential outliers were noted in both boxplots and residual plots. Analyses were repeated after

the outliers were removed, and significant results of the analysis remained unchanged. As there was no significant interaction between layer and body site for any FA (MANOVA, $p = 0.005$; ANOVAs, all $p \geq 0.002$), results of individual F-tests for each FA were interpreted.

Fatty acid variation with body site and blubber depth

Of the 35 FA examined, 25 did not vary significantly with body site (MANOVA, $p < 0.001$; ANOVAs, all $p \geq 0.002$). Multiple comparisons (Tukey's HSD, $\alpha = 0.05$) on the remaining ten FA did not reveal any sites, individually or in combination with others, that were consistently different from the rest (Figure 2.4). In fact, there was often much overlap among the groups of sites identified.

FA levels varied significantly with layer (MANOVA, $p < 0.001$; ANOVAs, all $p \leq 0.001$) with the exception of 14:0 ($F_{2,323} = 1.073$, $p = 0.343$), 16:2*n*6 ($F_{2,323} = 4.209$, $p = 0.016$) and 18:3*n*4 ($F_{2,323} = 2.503$, $p = 0.083$). Of the 32 other FA, 28 were present in significantly different amounts in all layers, typically forming a uni-directional gradient spanning the depth of the blubber (Tukey's HSD, $\alpha = 0.05$) (Figure 2.5). Levels of 16:0, 16:3*n*4, 16:4*n*1 and 18:4*n*3 in the middle layer were not significantly different from levels in either the inner or outer layers (Tukey's HSD, $\alpha = 0.05$).

Effects of age, body length, and sex on fatty acid stratification

Fatty acid stratification (i.e., SI.mean.ext) increased significantly with both age and length. When GLGs were used as a proxy for age, ANCOVA revealed that sex was not significant, thus a single equation described the relationship with age: SI.mean.ext = 0.29 + 0.01*GLGs ($F_{1,44} = 54.345$, $p < 0.001$, $R^2 = 0.55$) (Figure 2.6). When body length was used as the independent variable, ANCOVA revealed sex to be a significant factor; thus, separate equations describe the relationships with length. For females, SI.mean.ext = -0.26 + 0.25*length, while for males, SI.mean.ext = -0.39 + 0.25*length ($F_{2,92} = 23.295$, $p < 0.001$, $R^2 = 0.34$ for both) (Figure 2.7).

DISCUSSION

Fatty acid variation with body site

It is generally accepted that the blubber FA composition across much of the thorax of a cetacean is homogeneous. The inability to use FA to differentiate among the six body sites sampled on belugas in this study is consistent with results of investigations into the blubber of various species of odontocetes (Koopman et al. 1996, Krahn et al. 2004, Samuel & Worthy 2004) and mysticetes (Ruchonnet et al. 2006, Budge et al. 2008). The blubber on the portion of the thorax sampled in this, and most of the above listed studies, is non-structural, functioning primarily as a site of energy storage and insulation. Thus, the lipids found here are not constrained by the other "roles" commonly ascribed to specific types blubber, such as the locomotory roles of the tailstock and acting as a biological spring to increase

swimming efficiency (reviewed in Iverson 2002, 2009a). Although blubber along the thorax may also be important to hydrodynamic streamlining, this occurs as a matter of course in adipocyte filling in non-structural blubber. In their work on harbour porpoise, Koopman et al. (1996) found that the FA composition of blubber sampled at a dorsal position on the tailstock differed significantly from the three other, more anteriorly-located, sites. They attributed the observed differences in FA levels to the ability of the porpoise to selectively mobilize lipids from the anterior portion of the thorax and abdomen, leaving the lipid stores in the tailstock untouched, thus maintaining the streamlined shape of the tailstock for efficient locomotion.

The body sites and suite of FA analyzed in this study were selected to investigate blubber FA variation in the context of making dietary inferences. In contrast to the research cited above, the list of FA analyzed here was restricted to those commonly used to estimate diet with the QFASA model (the "extended" FA subset). Additionally, samples were only collected from the portion of the body most likely sampled on free-ranging animals. While this approach narrowed the scope of the work, the intent here was to specifically address whether samples collected from a range of body sites on an animal, as would be the case for those collected via remote-biopsy, would result in different diet estimates. The important conclusion is that samples collected from the portion of the thorax delimited by these six sites will likely lead to the same diet inferences.

Fatty acid variation with blubber depth

FA in beluga blubber clearly stratify with depth. The existence of stratification alone implies that blubber sampled at different depths will result in different diet inferences based on FA data. In addition, the distribution of the FA between layers supports the idea that it will be necessary to sample the innermost blubber layer in order to most accurately estimate diet – or at least to assess most recent diet. It remains to be tested whether sampling from the middle portion of the blubber layer produces "inaccurate" diet estimates or those representing a longer time period (see Chapter 4). FA belonging to the *n*-3 and *n*-6 classes must come from the diet as vertebrates are incapable of inserting double bonds at the *n*-3 and *n*-6 positions in the carbon chain (Cook 1985, Nelson 1992). Averaged across all body locations, *n*-3 FA accounted for 17.7% (by mass) of all FA in the inner layer, and 8.8% in the outer layer. Similarly, *n*-6 accounted for 2.3% of all FA in the inner layer, and 2.2% in the outer layer (Table 2.4). The increased presence of dietary FA in the inner blubber, coupled with increased presence of FA which could arise from biosynthesis in the middle and outer layers, suggests that the inner blubber should be sampled if the intention is to use FA to estimate diet.

While it is appropriate to suggest that blubber be collected from only the innermost layer, in reality this may be logistically impossible and potentially harmful to the animal being sampled. For animals that have died, and can be safely sampled onshore or on a boat, a full-depth blubber core is readily obtained. For animals to be sampled in the wild, the thickness of the blubber and skin will greatly influence the

portion of the blubber layer sampled. For example, blubber thickness may range from less than 1 cm in a small tropical cetacean (*Stenella attenuata*, Worthy & Edwards 1990), to roughly 5 cm in belugas (H. Smith, *unpublished data*), to 42 cm in bowhead whales (Mau 2004). Accordingly, the force required to pierce the skin and propel the biopsy dart through the blubber will be much less in tropical odontocetes than in any mysticete species. However, regardless of blubber thickness, and even if a remote biopsy tool can be developed to successfully retrieve a full-depth blubber core, the likelihood of accidentally sampling beyond the blubber (i.e., into the underlying muscle), or otherwise injuring the animal should be a primary concern. The most prudent course of action might be to simply collect a portion of the outer to middle blubber, while estimating the proportion of the thickness sampled – and perhaps using only the deepest subsection obtained. Clearly, analyzing blubber from various depths will influence diet inferences made using FA data, however, the extent to which this occurs must be investigated with QFASA modeling.

In this study, beluga blubber was divided into three equal layers. In the absence of any macroscopic characteristics to guide the division of blubber, an equal partitioning provides a tractable and repeatable approach. Here, beluga blubber was divided into thirds to correspond with the analysis of beluga blubber in the Saint Lawrence Estuary, Quebec, Canada (Nozeres et al. 2001).

Referring to specific layers in blubber is somewhat misleading, yet is done in the interest of making such analyses feasible. Many FA have been found in

quantities forming a unidirectional gradient across the depth of the blubber, with individual gradients running in either direction. Levels of particular FA may run from high to low levels either from inner to outer or from outer to inner blubber (e.g., Koopman et al. 1996, Smith & Worthy 2006, this study). In studies where blubber has been divided into 5 layers (minke whales (*Balaenoptera acutorostrata*), Olsen & Grahl-Nielsen 2003, bowhead whales, Budge et al. 2008), gradients in levels of many FA have been observed across the full depth of the blubber. That gradients continue to be observed as blubber is further divided suggests that blubber is not composed of well-defined layers as is the case with pinnipeds. However, this idea contrasts with (limited) observations that suggest lipid metabolism differs in specific portions (i.e., layers) of the blubber. For example, Koopman et al. (2003) found that levels of isovaleric acid (*i*-5:0) accumulated with age in greater quantities in the outer than in the inner blubber layer of harbour porpoises. In the same study, Koopman et al. noted that with the exception of *i*-5:0, levels of iso-acids (i.e., branched chain FA) were present in higher levels in the inner blubber of emaciated vs. normal-weight porpoises. Additionally, Olsen & Grahl-Nielsen (2003) found that differences in FA levels were proportionally greater between adjacent blubber layers closer to the skin (i.e., outer layers) than next to the muscle (i.e., inner layers). Clearly, classification of the heterogeneity in blubber FA with depth is not a simple matter. As further insight is gained into the processes influencing FA composition within the blubber, this can be used to inform the most appropriate method to sample

blubber for use in diet studies, as well as to evaluate potential impacts on diet estimates produced using FA analysis.

Effects of age, length, and sex on fatty acid stratification (SI)

The SI computed by Koopman et al. (1996) incorporates *i*-5:0, which has been shown to be highly stratified in a number of odontocete species, but does not come from diet. The SI computed here (SI.mean.ext) only incorporates FA commonly used to model diet with QFASA. Even in the absence of an influential FA like *i*-5:0, stratification in beluga blubber was found to increase with both "age" (assuming 1 GLG = 1 year of age) and body length. Thus, in the context of collecting blubber samples for diet studies, depth of sample collection will likely have an increasingly large influence on diet estimates as animals get older and/or larger. This could be due largely to sequestering of FA in less metabolically active outer-most layers with time (e.g., Koopman et al. 1996).

Alternatively, or in addition, since SI.mean.ext was computed using FA that can either come only from the diet, or are present in levels greatly influenced by the particular prey species consumed, this increase in stratification with beluga age and length could be interpreted as evidence for ontogenetic changes in foraging behaviour. Such differences in diet are likely as larger and more mature animals would be better able to catch preferred prey items owing to their larger body size and foraging experience. This agrees with the work of Loseto et al. (2009), who used a principal components analysis to explore the FA profiles of potential prey items and

belugas from the Beaufort Sea. The authors also examined the influence of body size on FA composition, and suggested that large whales preferred offshore Arctic cod, while smaller whales fed on prey items in nearshore habitats, including nearshore Arctic cod. Similarly, Seaman et al. (1982) found that younger belugas in the Bering and Chukchi Seas ate smaller saffron cod (*Eleginus gracilis*) than older belugas. Prior to correlating this pattern in FA variation with specific changes in diet, prey items likely to be consumed by eastern Chukchi Sea belugas must be collected and FA analyzed.

The effect of sex on the degree of stratification in the blubber is equivocal based on these results. Sex-specific differences in diet have been observed in belugas. Seaman et al. (1982) analyzed stomach contents of belugas from the Bering and Chuckchi Sea populations, and found that males ate proportionately more sculpins (species not identified) than females. Using stable isotopes, Lesage et al. (2001) found that in the Estuary and Gulf of St. Lawrence, male belugas occupied a higher trophic level and foraged more intensively on benthic prey species than females. In addition to the expectation that feeding on different prey species would result in different FA compositions for males and females, differential (and/or more extensive) lipid mobilization by females during lactation, as observed in hooded seals (*Cystophora cristata*) (Iverson et al. 1995b) and Weddell seals (*Leptonychotes weddellii*), might further exaggerate these differences. Specifically, I expected that the blubber of female belugas might be more stratified than male belugas owing to the greater mobilization and subsequent replenishing of FA from the inner, and more

metabolically active, portion of the blubber. While this pattern was observed when SI.mean.ext was regressed on body length (n = 95), this was not the case for the regression on GLGs (n = 46). These divergent results are likely confounded by the known differential growth trajectories for male and female belugas (O'Corry-Crowe et al. 1997). Because males grow faster than females and achieve a larger asymptotic body size at physical maturity (O'Corry-Crowe et al. 1997), body length is an imperfect index of age, particularly when making comparisons between sexes. For any given body length, a male will be younger on average than a similarly-sized female, potentially confounding detection of FA stratification differences presumed to be related to age. Furthermore, as in other species of cetaceans (Chivers 2002), eastern Chukchi Sea belugas attain sexual maturity prior to attaining physical maturity (Suydam 2009). More specifically, it is the attainment of age at first birth, and therefore first lactation (estimated to be 8.27 ± 2.88 years (mean ± SE) in this population of belugas (Suydam 2009)), as well as the number of calves successfully weaned, that would lead to increased FA stratification in females. As many of the female belugas sampled in this study were older than 8.27 years (Table 2.3), this failure to detect a significant effect of sex in the regression with GLGs could reflect a data issue. Not only was our sample of aged animals relatively small (n = 46), the number of animals of known-age animals was only n = 27. Repeating this analysis with a larger sample of known-age belugas is recommended.

Ecological vs. statistical significance of observed fatty acid variation

In this chapter, the significance of FA variation with both body site and blubber depth was evaluated statistically. While this represents an important step towards describing and understanding the distribution of FA within the blubber layer of belugas, it can only be used to suggest how this variation might influence diet estimates. In the fourth chapter of my dissertation, I examine the ecological significance of these results by producing diet estimates for the individual layers and body sites using QFASA.

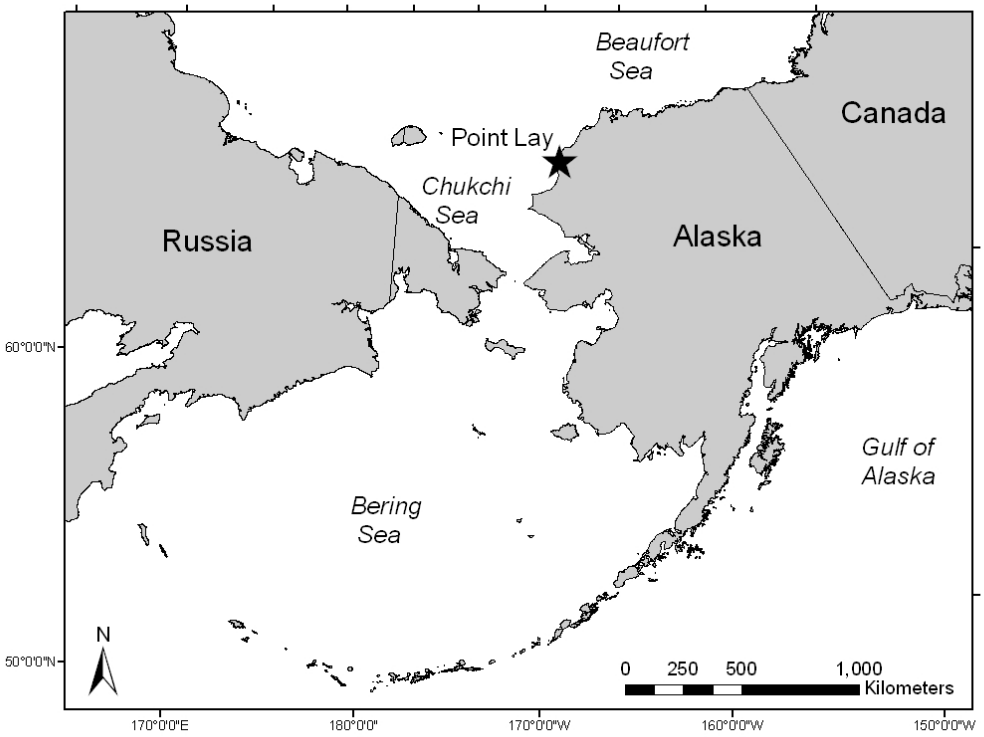

Figure 2.1. Location of the village of Point Lay, Alaska, where blubber samples were collected from belugas taken in the annual subsistence harvest.

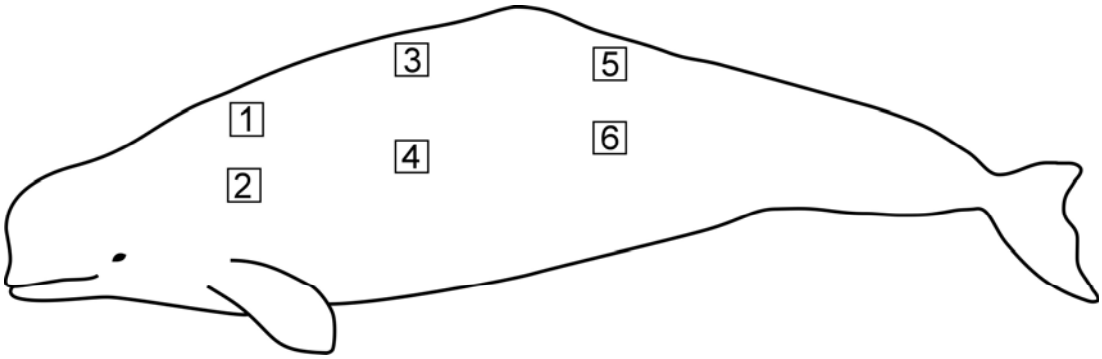

Figure 2.2. Approximate locations of blubber sample collection on the body of the beluga. Full-depth blubber samples (from skin to muscle) were collected from each individual (n = 20) at all six body sites.

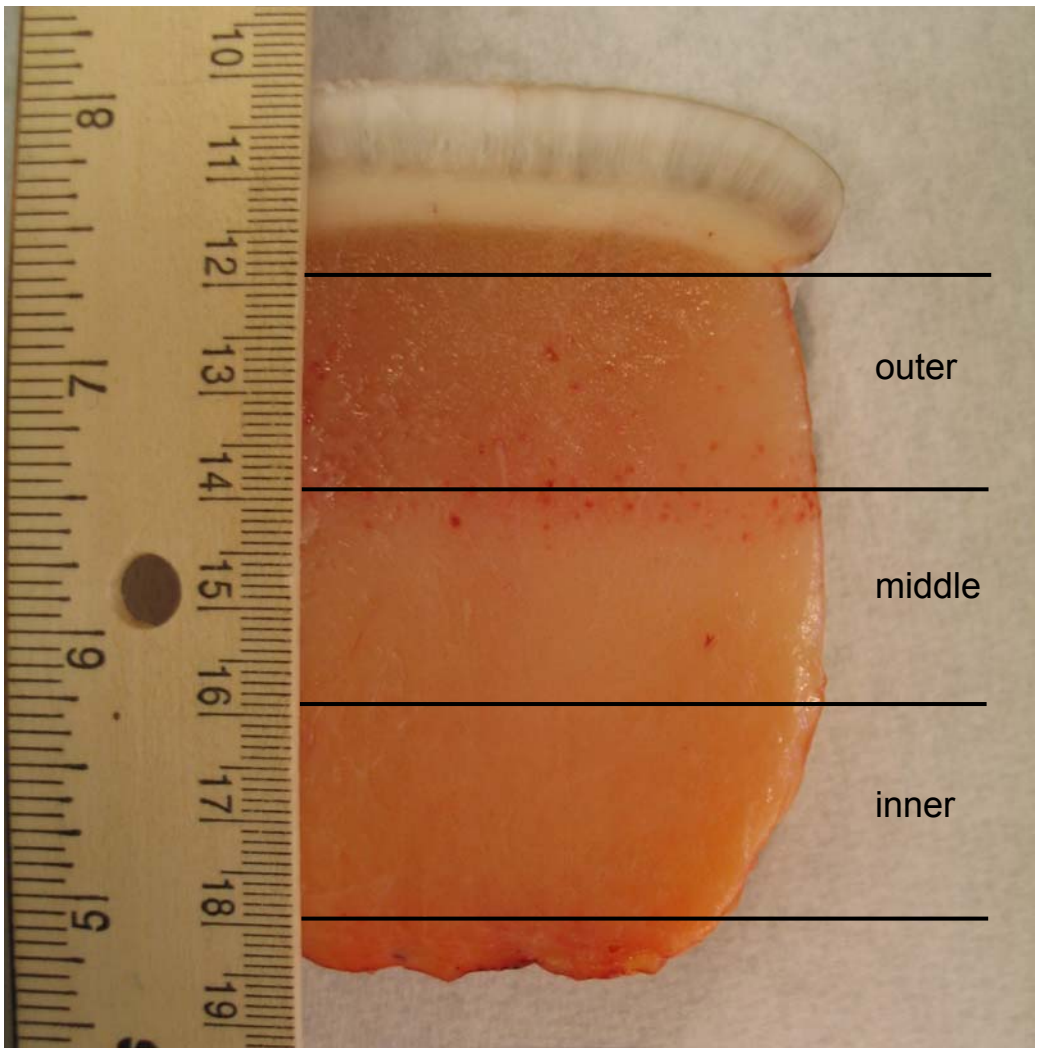

Fig. 2.3. Division of blubber into three equal layers prior to fatty acid composition analysis. Horizontal lines indicate where blubber was cut with scalpel. Ruler at left shows units in cm (numbered intervals in scale on right side of ruler).

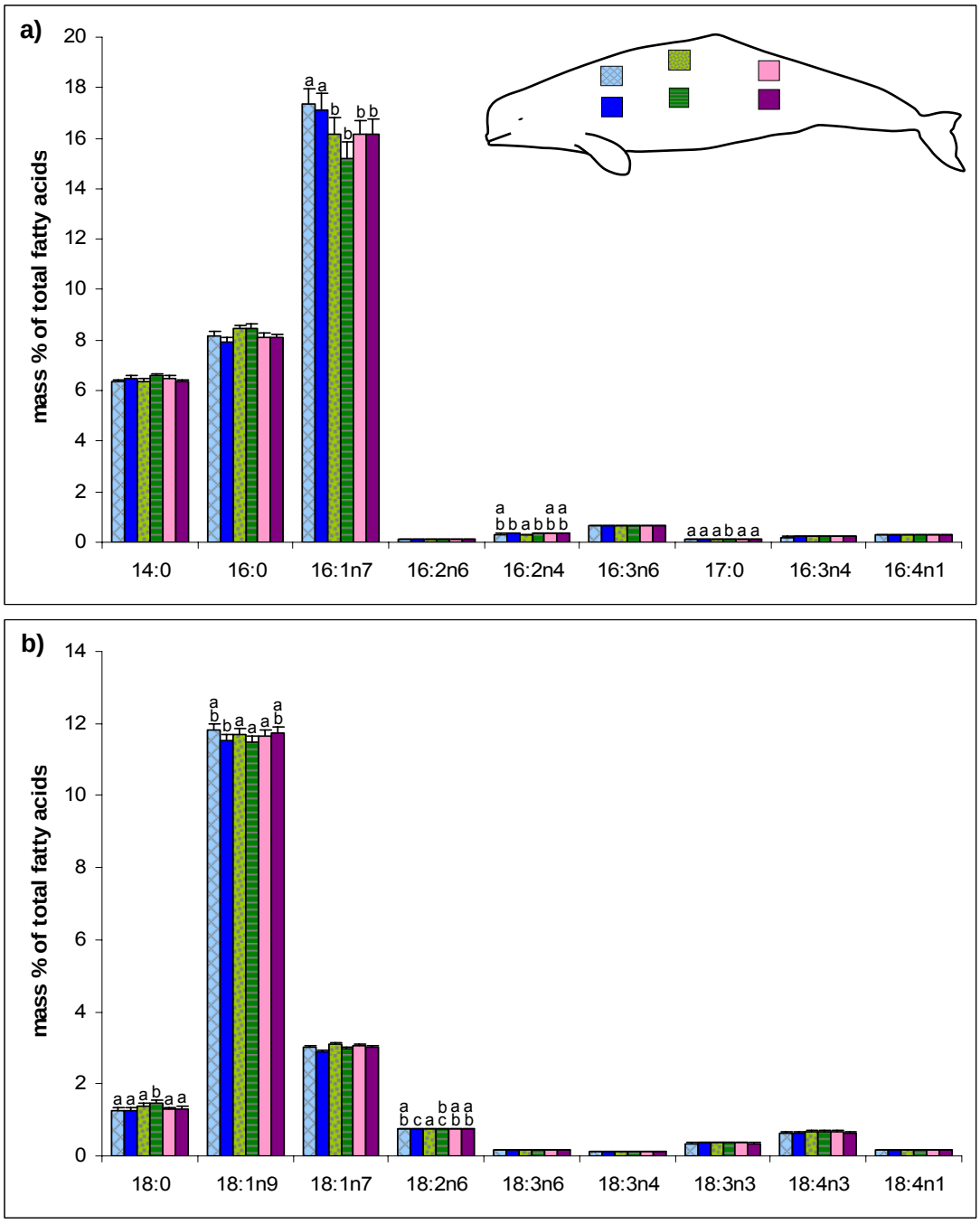

Figure 2.4. a-b. Mass % of "extended" fatty acids (FA) in blubber at six sites from *n* = 20 adult belugas. Bars are means (full blubber depth) ± SE. For FA where site was significant (ANOVA, $p < 0.0014$ (Bonferroni corrected)), bars sharing lower-case letters were not significantly different (Tukey's HSD, $\alpha = 0.05$). Significance was assessed using transformed data ($x_{trans} = \ln(x_i/c_r)$, where x_{trans}= transformed FA, x_i = mass % of FA to be transformed, and c_r = mass % of 18:1*n*7 (reference FA).

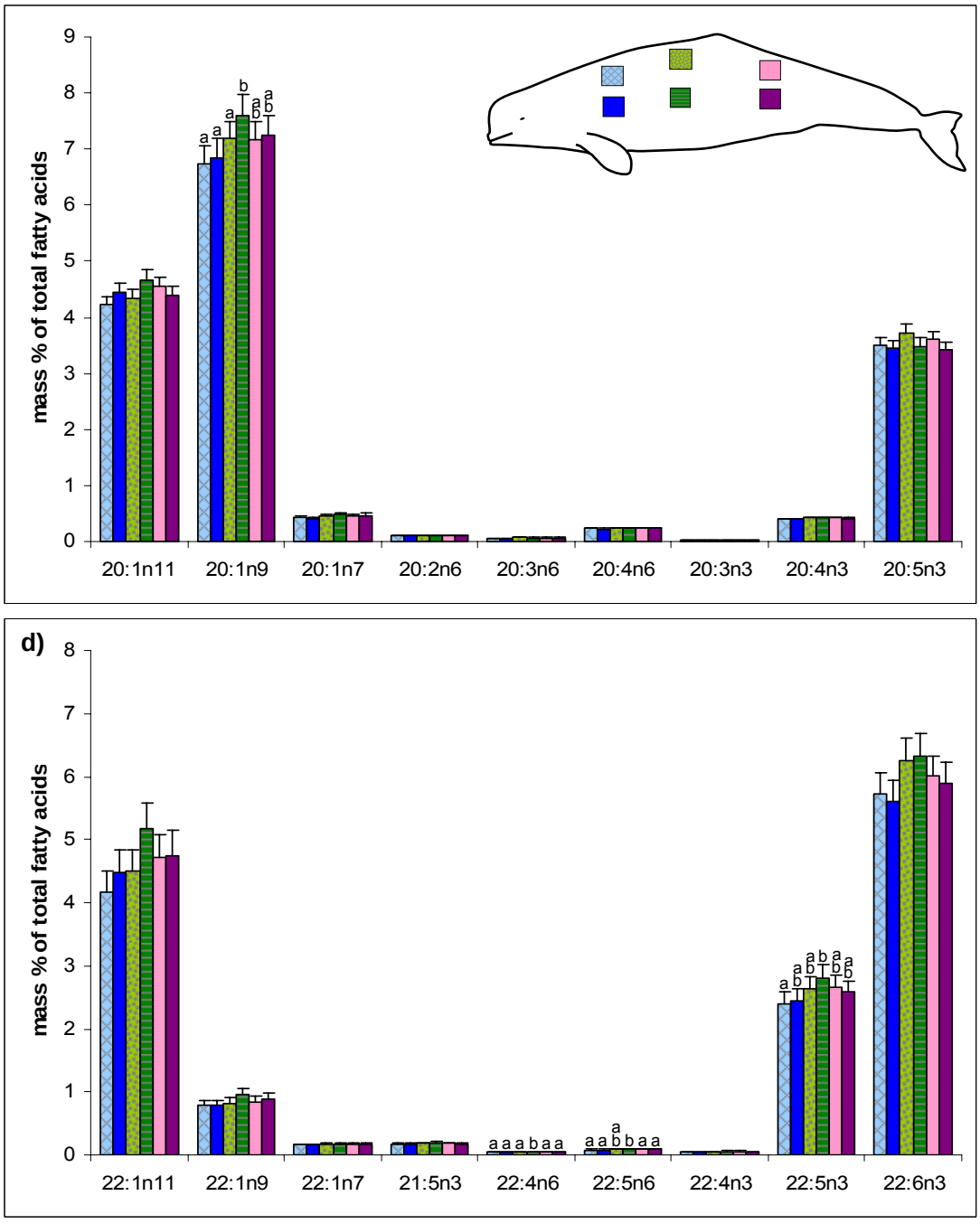

Figure 2.4. c-d. Mass % of "extended" fatty acids (FA) in blubber at six sites from *n* = 20 adult belugas. Bars are means (full blubber depth) ± SE. For FA where site was significant (ANOVA, $p < 0.0014$ (Bonferroni corrected)), bars sharing lower-case letters were not significantly different (Tukey's HSD, $\alpha = 0.05$). Significance was assessed using transformed data ($x_{trans} = \ln(x_i/c_r)$, where x_{trans}= transformed FA, x_i = mass % of the FA to be transformed, and c_r = mass % of 18:1*n*7 (reference FA).

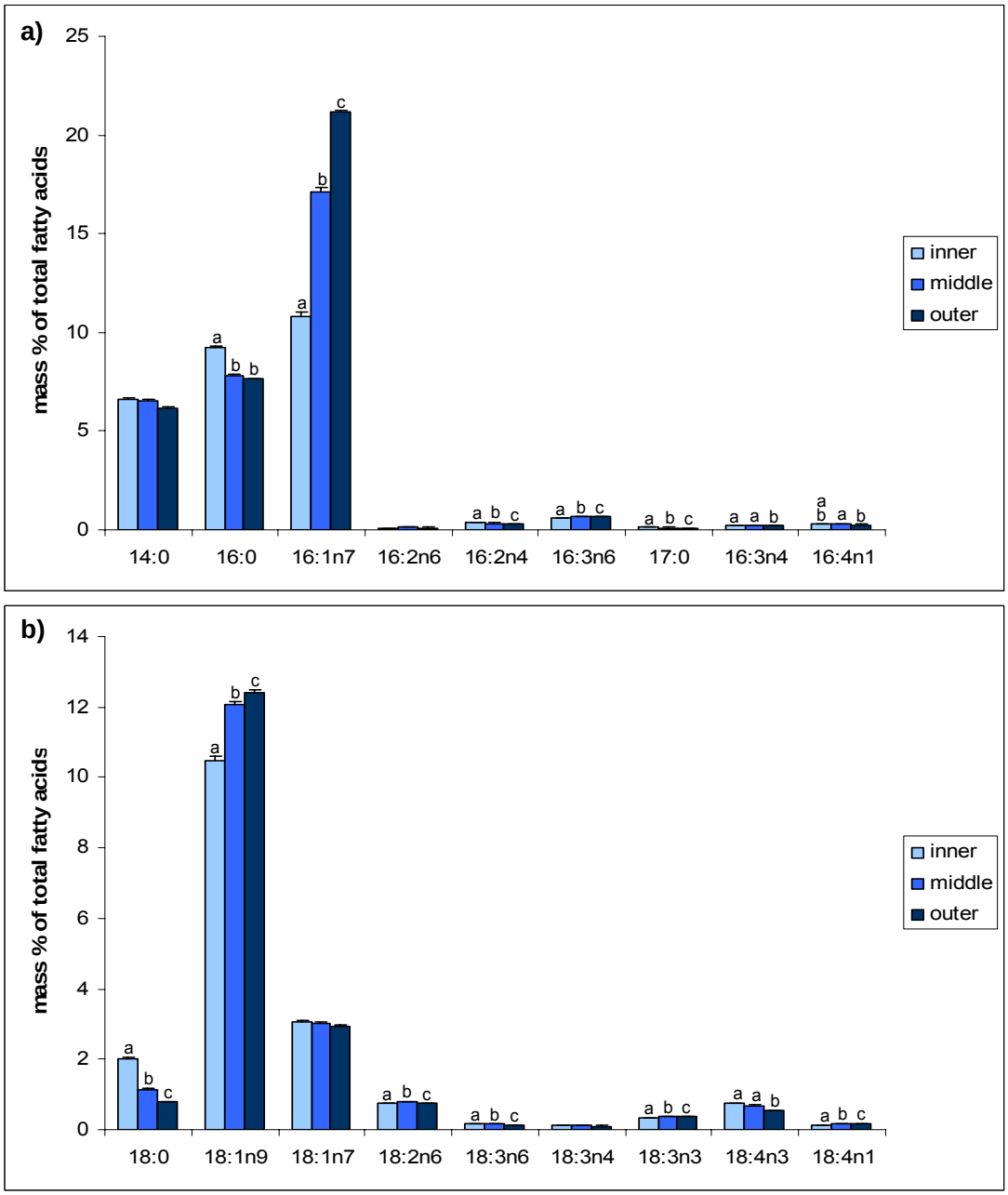

Figure 2.5. a-b. Mass % of "extended" fatty acids (FA) for blubber divided equally into inner, middle and outer layers for $n = 20$ adult belugas. Blubber was collected from six sites on the body (see Figure 2.2), and bars are means (across all six sites) ± SE bars. For FA where layer was significant (ANOVA, $p < 0.0014$ (Bonferroni corrected)), bars with the same lower-case letter were not significantly different (Tukey's HSD, $\alpha = 0.05$). Significance was assessed using transformed data ($x_{trans} = \ln(x_i/c_r)$, where x_{trans}= transformed FA, x_i = mass % of the FA to be transformed, and c_r = mass % of 18:1*n*7 (reference FA).

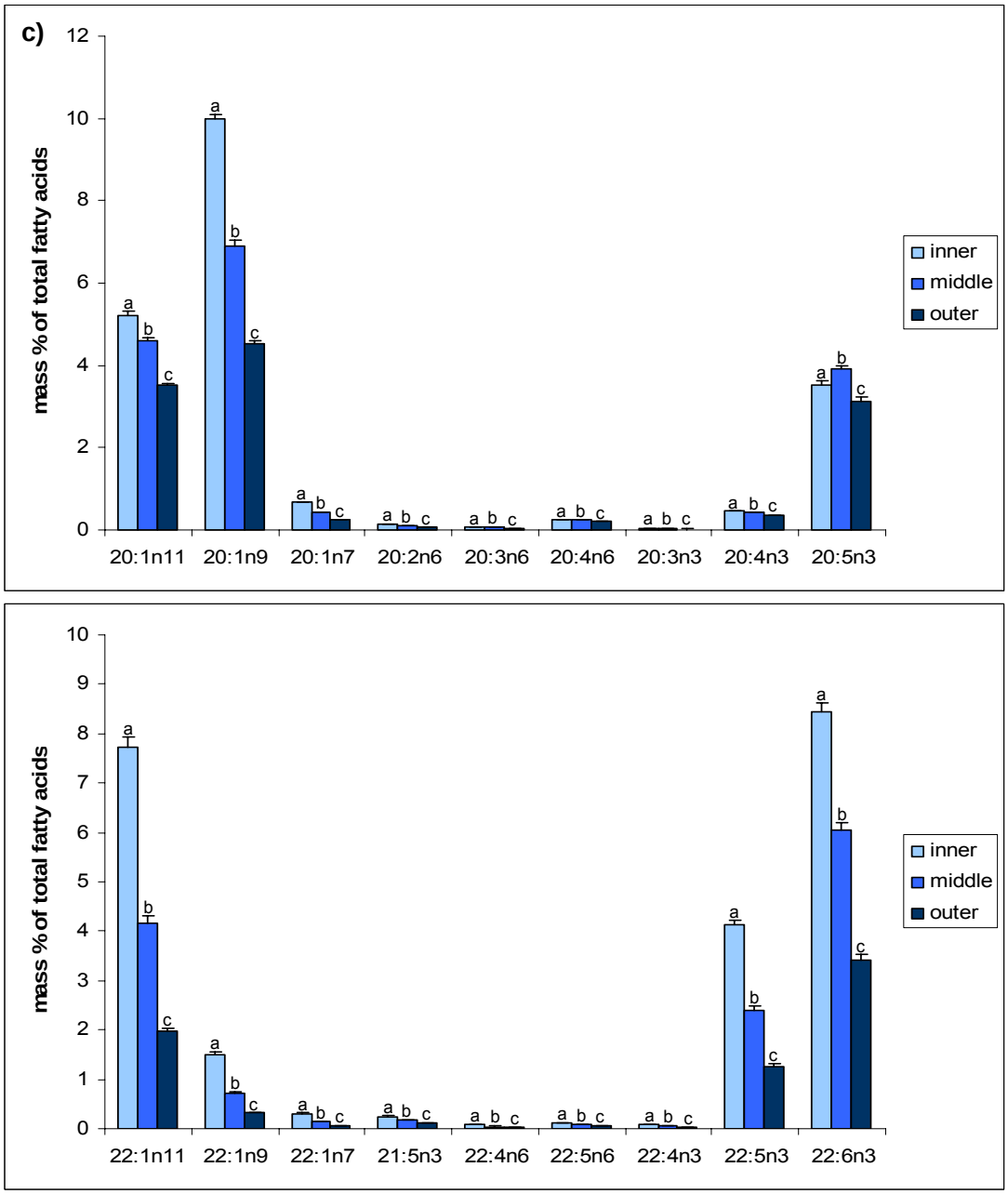

Figure 2.5. c-d. Mass % of "extended" fatty acids (FA) for blubber divided equally into inner, middle and outer layers for $n = 20$ adult belugas. Blubber was collected from six sites on the body (see Figure 2.2), and bars are means (across all six sites) ± SE bars. For FA where layer was significant (ANOVA, $p < 0.0014$ (Bonferroni corrected)), bars with the same lower-case letter were not significantly different (Tukey's HSD, $\alpha = 0.05$). Significance was assessed using transformed data ($x_{trans} = \ln(x_i/c_r)$, where x_{trans}= transformed FA, x_i = mass % of the FA to be transformed, and c_r = mass % of 18:1*n*7 (reference FA).

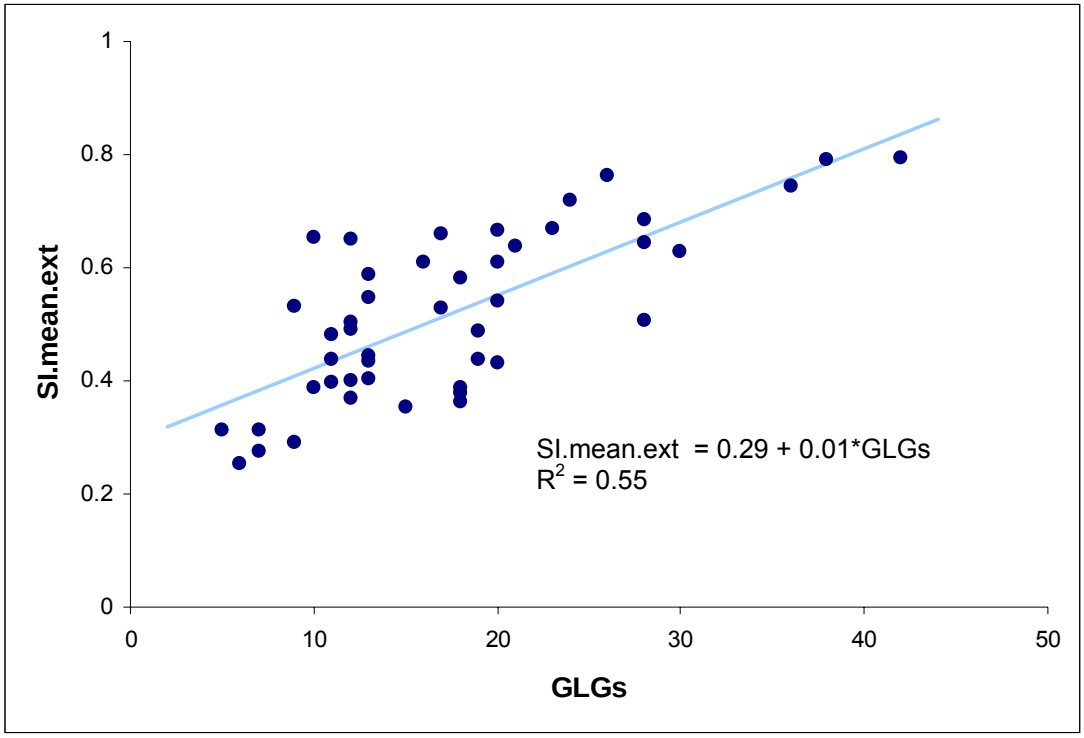

Figure 2.6. Relationship between mean stratification of the 35 fatty acids (FA) in the extended FA subset (SI.mean.ext) and "age" (represented by GLGs, growth layer groups, in teeth) in n=46 belugas. Sex was not found to be a significant factor (ANCOVA, $F_{1,43} = 2.759$, $p=0.104$); thus, a simple regression was performed ($F_{1,44} = 54.345$, $p < 0.001$).

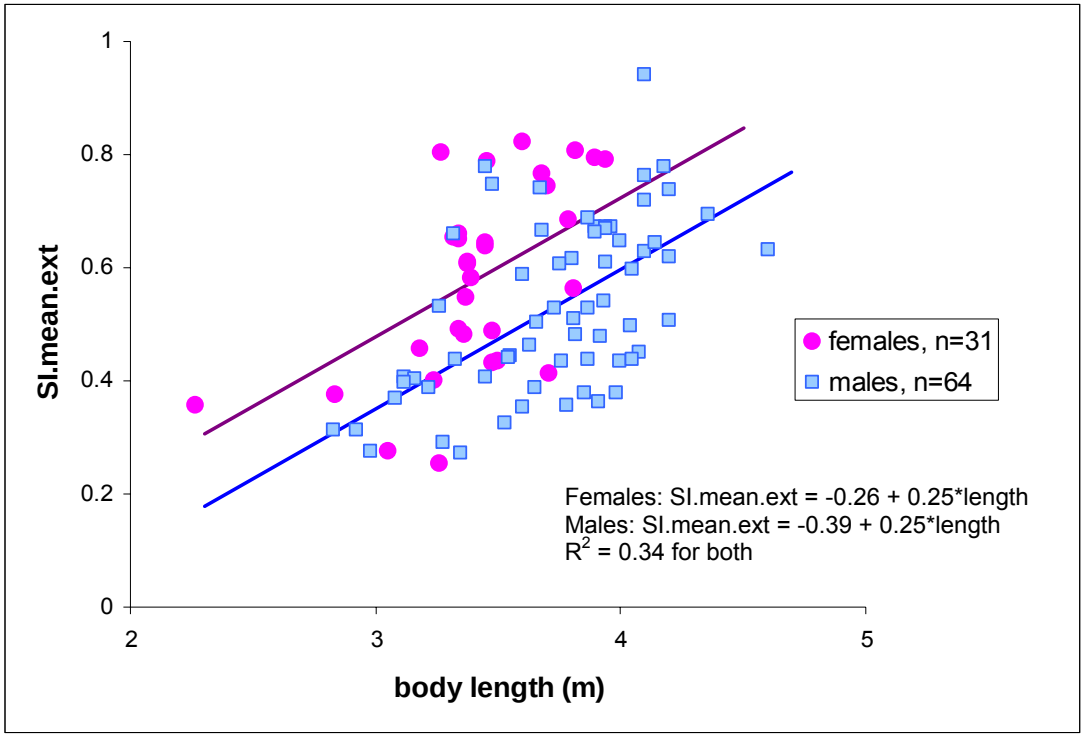

Figure 2.7. Relationship between mean stratification of the 35 fatty acids (FA) in the extended FA subset (SI.mean.ext) and body length in n=95 belugas. Significant relationships were found for both sexes.

Table 2.1. Fatty acid (FA) stratification documented in the blubber of cetaceans. Note that the blubber has been divided into a variety of layers based on a number of criteria depending on the study. Given the various subsets of FA used and statistical methods employed, the extent to which the composition varies among layers in different species is difficult to compare.

Species	***Layers***	***Citation***
Odontocetes		
Harbour porpoise (*Phocoena phocoena*)	2, equal thickness	Koopman et al. 1996
Amazon River dolphin (*Inia geoffrensis*)	2, equal thickness	Ackman et al. 1971
Common dolphin (*Delphinus* spp)	3, equal thickness	Smith & Worthy 2006
Northern bottlenose whale (*Hyperoodon ampullatus*)	3, equal thickness	Hooker et al. 2001
Killer whale (*Orcinus orcas*) & beluga (*Delphinapterus leucas*)	various thicknesses, based on blubber thickness and "length" of sample collected via remote biopsy dart	Krahn et al. 2004
Bottlenose dolphin (*Tursiops truncatus*)	3, equal thickness	Samuel & Worthy 2004
Various odontocetes	2, equal thickness	Koopman 2007
Mysticetes		
Fin whale (*Balaenoptera physalus*)	3, equal thickness	Ackman & Eaton 1966
Fin whale (*Balaenoptera physalus*)	3, unequal thickness (based on macroscopic differences)	Lockyer et al. 1984
Fin whale (*Balaenoptera physalus*)	3, equal thickness	Ruchonnet et al. 2006
Bowhead whale (*Balaena mysticetus*)	5, equal thickness	Budge et al. 2008
Minke whale (*Balaenoptera acutorostrata*)	5, equal thickness	Olsen & Grahl-Nielsen 2003

Table 2.2. Individuals used to evaluate the effect of sampling location (blubber depth and body location) on fatty acid composition in beluga blubber. Belugas were sampled at Point Lay, Alaska in July 2002. Skin colour was used as a proxy for age; young animals are grey and turn white as they get older. Colour codes: G = grey, GW = grey-white, W = white. GW animals had a mean length of 351 cm, and W animals had a mean length of 375 cm.

Sex	Total Length (cm)	Fluke Width (cm)	Skin Colour	Animal ID#
Female	284	67	G	LDL4302
Female	332	76	GW	LDL1002
Female	334	82	GW	LDL4002
Female	336	78	GW	LDL1102
Female	327	74	W	LDL3402
Female	338	70	W	LDL3202
Female	360	86	W	LDL0702
Female	368	81	W	LDL0602
Female	379	81	W	LDL0902
Female	381	79	W	LDL2902
Male	367	77	GW	LDL1402
Male	387	90	GW	LDL0802
Male	326	81	W	LDL4102
Male	345	85	W	LDL4202
Male	348	74	W	LDL4402
Male	390	83	W	LDL4502
Male	410	99	W	LDL0502
Male	418	104	W	LDL1502
Male	420	99	W	LDL1302
Male	436	100	W	LDL2802

Table 2.3. Samples used to evaluate the effects of age and body length on the degree of fatty acid stratification in beluga blubber. Samples were collected at Point Lay, Alaska in June/July 2002, 2005-2008. All samples were collected from body site #4 (see Figure 2.2). Body length was measured in a straight line from the tip of the snout to the notch in the tail flukes. Age was determined by counting growth layer groups (GLGs) in teeth. Actual age was estimated when the neonatal line was present on the teeth, minimum age was estimated when the neonatal line was absent.

	Females			**Males**		
	mean	range	*n*	mean	range	*n*
all belugas (n=95)						
length (m)	3.4	2.3 – 3.9	31	3.7	2.8 – 4.6	64
belugas with actual age estimates (n=25)						
age (GLGs)	13	6 – 20	13	11	5 – 19	12
length (m)	3.3	3.1 – 3.5		3.3	2.8 – 4.1	
belugas with minimum age estimates (n=21)						
age (GLGs)	32	21 – 42	6	20	13 – 30	15
length (m)	3.7	3.5 – 3.9		3.9	3.6 – 4.2	

Table 2.4. Fatty acid (FA) composition of inner, middle, and outer blubber of individuals sampled at Point Lay in 2002 (*n*=20). Values are mean ± SE, mass % of all 70 FA identified, across all six body locations. Selected "extended" FA used in data analysis, are in bold. FA are named using the shorthand nomenclature of C:Dn-x, where C is the number of carbon atoms, D is the number of double bonds, and n-x denotes the position of the first double bond relative to the terminal methyl end of the FA. Branched chain FA are prefixed by *i = iso* or *ai = anteiso*.

Fatty Acid	**Inner**	**Middle**	**Outer**
saturated fatty acids (SFA)			
i-4:0	0.03 ± 0.00	0.17 ± 0.01	0.47 ± 0.01
4:0	0.06 ± 0.00	0.06 ± 0.00	0.09 ± 0.00
i-5:0	1.46 ± 0.11	4.91 ± 0.21	10.28 ± 0.25
6:0	0.01 ± 0.00	0.01 ± 0.00	0.02 ± 0.00
8:0	0.01 ± 0.00	0.02 ± 0.00	0.02 ± 0.00
10:0	0.07 ± 0.00	0.15 ± 0.00	0.20 ± 0.00
i-11:0	0.28 ± 0.00	0.30 ± 0.00	0.32 ± 0.01
11:0	0.01 ± 0.00	0.02 ± 0.00	0.03 ± 0.00
i-12:0	0.02 ± 0.00	0.09 ± 0.00	0.24 ± 0.01
12:0	0.44 ± 0.03	1.00 ± 0.04	1.14 ± 0.03
i-13:0	0.06 ± 0.00	0.19 ± 0.01	0.37 ± 0.01
13:0	0.04 ± 0.00	0.06 ± 0.00	0.06 ± 0.00
i-14:0	0.05 ± 0.00	0.12 ± 0.00	0.29 ± 0.01
14:0	6.62 ± 0.05	6.56 ± 0.05	6.18 ± 0.06
i-15:0	0.37 ± 0.01	0.59 ± 0.02	1.20 ± 0.03
ai-15:0	0.11 ± 0.00	0.15 ± 0.00	0.16 ± 0.00
15:0	0.35 ± 0.00	0.36 ± 0.00	0.38 ± 0.00
i-16:0	0.12 ± 0.00	0.21 ± 0.01	0.59 ± 0.01
16:0	9.22 ± 0.13	7.80 ± 0.07	7.63 ± 0.05
7Me16:0	0.43 ± 0.01	0.48 ± 0.01	0.49 ± 0.00
17:0	0.16 ± 0.00	0.11 ± 0.00	0.09 ± 0.00
18:0	2.03 ± 0.04	1.15 ± 0.03	0.79 ± 0.01
20:0	0.10 ± 0.00	0.07 ± 0.00	0.04 ± 0.00
∑SFA	22.05 ± 0.20	24.56 ± 0.25	31.09 ± 0.30
monounsaturated fatty acids (MUFA)			
5:1	0.13 ± 0.00	0.10 ± 0.00	0.09 ± 0.00
14:1n7	0.20 ± 0.02	0.54 ± 0.03	0.59 ± 0.03
14:1n5	0.47 ± 0.03	1.51 ± 0.06	2.32 ± 0.04

Table 2.4 continued

Fatty Acid	Inner	Middle	Outer
15:1n8	0.03 ± 0.00	0.04 ± 0.00	0.04 ± 0.00
15:1n6	0.03 ± 0.00	0.07 ± 0.00	0.11 ± 0.00
16:1n11	0.22 ± 0.00	0.21 ± 0.00	0.19 ± 0.00
16:1n9	0.64 ± 0.03	1.18 ± 0.04	1.27 ± 0.03
16:1n7	10.83 ± 0.23	17.08 ± 0.28	21.15 ± 0.12
16:1n5	0.14 ± 0.00	0.17 ± 0.00	0.31 ± 0.01
17:1	0.17 ± 0.00	0.25 ± 0.00	0.28 ± 0.00
18:1n11	3.19 ± 0.06	3.68 ± 0.03	3.41 ± 0.02
18:1n9	10.49 ± 0.10	12.07 ± 0.10	12.42 ± 0.06
18:1n7	3.06 ± 0.03	3.04 ± 0.03	2.93 ± 0.04
18:1n5	0.70 ± 0.01	0.59 ± 0.01	0.48 ± 0.01
20:1n11	5.21 ± 0.12	4.58 ± 0.09	3.53 ± 0.04
20:1n9	9.98 ± 0.13	6.90 ± 0.13	4.53 ± 0.08
20:1n7	0.67 ± 0.02	0.42 ± 0.01	0.25 ± 0.00
22:1n11	7.73 ± 0.20	4.18 ± 0.14	1.99 ± 0.04
22:1n9	1.51 ± 0.06	0.72 ± 0.02	0.32 ± 0.01
22:1n7	0.31 ± 0.01	0.14 ± 0.00	0.06 ± 0.00
24:1n9	0.48 ± 0.01	0.23 ± 0.01	0.12 ± 0.01
∑MUFA	56.19 ± 0.49	57.70 ± 0.20	56.40 ± 0.10
polyunsaturated fatty acids (PUFA)			
16:2n6	0.11 ± 0.00	0.12 ± 0.00	0.11 ± 0.00
16:2n4	0.37 ± 0.00	0.34 ± 0.00	0.30 ± 0.00
16:3n6	0.58 ± 0.01	0.68 ± 0.01	0.69 ± 0.01
16:3n4	0.24 ± 0.01	0.23 ± 0.00	0.19 ± 0.00
16:4n1	0.30 ± 0.01	0.30 ± 0.01	0.26 ± 0.00
18:2d5,11	0.04 ± 0.00	0.03 ± 0.00	0.02 ± 0.00
18:2n7	0.07 ± 0.00	0.07 ± 0.00	0.08 ± 0.00
18:2n6	0.75 ± 0.00	0.79 ± 0.00	0.74 ± 0.00
18:3n6	0.18 ± 0.00	0.16 ± 0.00	0.14 ± 0.00
18:3n4	0.11 ± 0.00	0.11 ± 0.00	0.10 ± 0.00
18:3n3	0.34 ± 0.01	0.36 ± 0.00	0.37 ± 0.00
18:4n3	0.73 ± 0.02	0.68 ± 0.02	0.54 ± 0.01
18:4n1	0.14 ± 0.00	0.15 ± 0.00	0.16 ± 0.00
20:2n9	0.19 ± 0.00	0.14 ± 0.00	0.10 ± 0.00

Table 2.4 continued

Fatty Acid	Inner	Middle	Outer
20:2n6	0.14 ± 0.00	0.10 ± 0.00	0.08 ± 0.00
20:3n6	0.08 ± 0.00	0.07 ± 0.00	0.05 ± 0.00
20:4n6	0.24 ± 0.00	0.24 ± 0.00	0.22 ± 0.01
20:3n3	0.04 ± 0.00	0.03 ± 0.00	0.02 ± 0.00
20:4n3	0.46 ± 0.01	0.42 ± 0.01	0.35 ± 0.01
20:5n3	3.53 ± 0.11	3.92 ± 0.09	3.14 ± 0.09
21:5n3	0.25 ± 0.01	0.18 ± 0.01	0.11 ± 0.00
22:4n6	0.08 ± 0.00	0.04 ± 0.00	0.02 ± 0.00
22:5n6	0.13 ± 0.00	0.09 ± 0.00	0.05 ± 0.00
22:4n3	0.09 ± 0.00	0.05 ± 0.00	0.02 ± 0.00
22:5n3	4.12 ± 0.09	2.39 ± 0.08	1.26 ± 0.05
22:6n3	8.45 ± 0.17	6.04 ± 0.15	3.42 ± 0.11
∑PUFA(all)	21.76 ± 0.40	17.74 ± 0.35	12.51 ± 0.30
∑PUFA(n-3)	17.68 ± 0.37	13.71 ± 0.33	8.84 ± 0.28
∑PUFA(n-6)	2.30 ± 0.01	2.37 ± 0.01	2.20 ± 0.01

CHAPTER THREE

ISOVALERIC ACID: EVIDENCE FOR LOCAL BIOSYNTHESIS AND THE INFLUENCE OF AGE AND SEX

INTRODUCTION

Lipid metabolism is poorly understood in marine mammals, particularly in odontocetes. Some species are found to possess relatively high concentrations of unusual components in both their blubber and other fatty tissues such as the melon and mandibular fats. One such component is isovaleric acid (*i*-5:0), a short, branched-chain fatty acid (FA) biosynthesized in only a few species (Koopman 2007).

Koopman et al. (1996) were the first to report extremely high levels of *i*-5:0 in the outer blubber layer of harbour porpoise (*Phocoena phocoena*) inhabiting cold Atlantic waters, and found that the amount of *i*-5:0 present was directly correlated with age. Isovaleric acid cannot arise from diet as it is not found in any marine prey. Instead it is biosynthesized by the porpoise itself. The findings of Koopman et al. (1996) suggest two primary conclusions. 1) i-5:0 may be sequestered with age and may have an important role in thermoregulation. It was present in high levels (2-27%, by mass) in the outermost blubber, and appears to provide four times the quality of insulation compared to the innermost blubber layer, where it is present in much lower levels. 2) Because of such predictable sequestering in the outer layer, it

might be possible to use *i*-5:0 in outer blubber to estimate age of free-ranging animals.

Isovaleric acid is not present in all cetaceans or even all odontocetes (Koopman et al. 2003). It is absent in right (*Eubalaena spp.*), sperm (*Physeter macrocephalus*), bottlenose (*Hyperoodon sp.*) and beaked (F. Ziphiidae) whales. However, it is present in the beluga (*Delphinapterus leucas*), another relatively small body-sized odontocete. Although previous work on belugas has been limited, moderate levels of *i*-5:0 were found in belugas from the Gulf of Saint Lawrence and relatively high levels in belugas from Alaska, suggesting that the hypothesis of its relation to cold water and thermoregulation may be supported (S. Iverson, *unpublished data*). In addition, preliminary evidence suggested that the levels of *i*-5:0 were directly correlated with age in Alaska belugas, explaining 69% of the variation in the 12 animals examined, of ages 8-46 years.

To date, variation in *i*-5:0 levels in blubber has only been thoroughly investigated in the harbour porpoise (Koopman et al. 1996). Isovaleric acid levels have been measured in a variety of odontocete tissues, but much of this work has focused on acoustic fat bodies (Varanasi & Malins 1972, Litchfield et al. 1975, Gardner & Varanasi 2003, Koopman et al. 2006). Some studies have analyzed tissues including liver and muscle (Litchfield et al. 1975, Morii 1980, Koopman et al. 2003); however, most analytical techniques employed were less sophisticated than those currently available. As well, most previous studies did not quantify the suite of

individual FA frequently identified in lipid samples today; thus, limiting the ability to make inferences regarding lipid metabolism in these species.

Almost nothing is known about the metabolism of *i*-5:0 in odontocetes. Malins et al. (1972) demonstrated the incorporation of isovaleroyl CoA, a catabolite of leucine, into triacylglycerols in bottlenose dolphin (*Tursiops truncatus*) melon, and Morii and Kaneda (1982) demonstrated the biosynthesis of branched-chain FA from leucine in the melon and blubber of the striped dolphin (*Stenella caeruleoalba*). On the basis of its toxicity to animals, including humans (Tanaka et al. 1966, Budd et al. 1967), when present in the bloodstream, it has been hypothesized that *i*-5:0 is only biosynthesized in the tissues where it is found.

Using FA to make qualitative and quantitative inferences about the diet of various pinnipeds has become increasingly common (Iverson 2009b), and some researchers have used FA data to make qualitative inferences about beluga diet (Dahl et al. 2000, Nozeres et al. 2001, Loseto et al. 2008, Loseto et al. 2009). It is understood that the FA composition of marine mammal blubber is constrained by its numerous functions including energy storage, body streamlining, and thermoregulation (Iverson 2002). Given that relatively high levels of *i*-5:0 have been measured in beluga blubber (Koopman et al. 2003), an increased understanding of lipid metabolism in this species is needed in order to better appreciate the extent to which this branched-chain FA may interfere with estimating diet using methods such as quantitative fatty acid signature analysis (QFASA) (Iverson et al. 2004).

Chapter objectives

The goal of this chapter was to determine the presence and abundance of *i*-5:0 in acoustic and non-acoustic tissues in the beluga, and to assess whether this FA is likely biosynthesized within the blubber itself. I also investigated the effects of age and sex on *i*-5:0 levels and degree of stratification in thorax blubber.

METHODS

Sample collection

Tissue samples were collected from belugas harvested by subsistence hunters in the village of Point Lay, Alaska (Figure 2.1) in late June/early July in 2002 and in each year from 2005 to 2008. Approximately 20 animals were sampled each year. The majority of the animals sampled were relatively large males, despite sampling all available small and/or female animals, because of size- and sex-specific biases in the hunts (Suydam 2009).

Belugas were sampled with the permission and cooperation of the elders and people of Point Lay, Alaska. Samples were collected under the U.S. Marine Mammal Protection Act (MMPA) Permit No. 782-1694-02 to the National Marine Mammal Laboratory.

Presence of i-5:0 in multiple tissues

A suite of tissues (blubber, melon, liver, and heart) were collected from 13 belugas (Table 3.1). Full-depth blubber cores were collected from both a mid-lateral

position on the thorax, just anterior to the dorsal ridge, and from the tailstock (Figure 3.1). Blubber cores had skin attached, and were roughly 5 cm x 5 cm in cross section, allowing samples to be trimmed of freezer-burned tissue immediately prior to lipid extraction in the lab. Blubber was divided into three equal horizontal layers: inner (adjacent to muscle), middle and outer (adjacent to skin) (Figure 2.3). Melon tissue samples were roughly 10 cm x 10 cm x 10 cm, and were collected from as near the centre of the melon as could be determined in the field. Samples of heart and liver were roughly 5 cm x 5 cm x 5 cm. All tissues were collected and frozen at -20 °C within approximately 24 hours of death.

Effects of age and sex on i-5:0 levels and stratification

Full-depth blubber cores were collected from the thorax (n = 95), as described above. Blubber was divided into three equal layers as was described in Chapter 2 (Figure 2.3). Straight-line body length, from the tip of the snout to the notch in the flukes, was measured to the nearest cm for all animals. Half of the lower jaws, with teeth to be used for age determination, were collected from 46 of these animals (Table 2.3).

Lipid analysis

Lipids were extracted using the Folch procedure (Folch et al. 1957), modified as described by Budge et al. (2006). Briefly, for blubber and melon, lipids were mashed from 0.5 g pieces of tissue into 2:1 chloroform:methanol with 0.01 % BHT

(v/v/w) added as an anti-oxidant. This mixture was washed with 0.7 % NaCl, and centrifuged to separate the lipid-containing chloroform layer. The chloroform layer was then dried with anhydrous sodium sulfate, and evaporated under nitrogen to yield the lipid extract. For heart and liver, tissue was homogenized, and 1.5 g of homogenate was soaked in 2:1 chloroform:methanol with 0.01 % BHT. The mixture was then filtered through plain filter paper, and the resulting filtrate was washed with 0.88 % NaCl. The lipid-containing chloroform layer was separated by means of centrifugation, dried with anhydrous sodium sulfate, and evaporated under nitrogen as was done for blubber and melon samples.

Fatty acid butyl esters (FABE) were prepared instead of the more commonly used fatty acid methyl esters (FAME) to allow for the quantification *i*-5:0. FABE were prepared by heating extracted lipid in hexane with 10 % boron trifluoride (BF_3) in butanol at 100 °C for one hour (Budge et al. 2006). Lastly, FABE in hexane were dried with anhydrous sodium sulfate.

FABE were analyzed in duplicate using temperature programmed gas-liquid chromatography. Separations were carried out on an Agilent HP 6890 gas chromatograph (GC) fitted with a 30 m × 0.25 mm ID flexible fused silica column coated with (50 %-Cyanopropyl)-methylpolysiloxane (0.25 μm film thickness; DB-23; Agilent Technologies, USA). Hydrogen was used as the carrier gas, and the gas line was equipped with oxygen and hydrocarbon scrubbers. Oven programming from Budge et al. (2006) was used. The initial oven temperature of 65 °C was held for 2 min, ramped to 165 °C at 20 °C/min, held for 0.4 min, ramped to 215 °C at 2

°C/min, held for 3 min, ramped to 235 °C at 5 °C/min, and then ramped to 240 °C at 25 °C and held for 0.5 min, producing a total runtime of approximately 40 minutes. The GC was linked to a flame-ionization detector (FID). The GC-FID was controlled by, and peaks identified and integrated using ChemStation software (Rev A.10.02, Agilent Technologies, USA). FA were identified by comparison with commercially prepared and known reference mixtures. Individual FA were converted to mass percent of total FA identified by applying theoretical response factors (TRF) relative to 18:0 (Ackman & Sipos 1964, Ulberth et al. 1999). FA are named using the shorthand nomenclature of C:Dn-x, where C is the number of carbon atoms, D is the number of double bonds, and n-x denotes the position of the first double bond relative to the terminal methyl end of the FA.

Age determination

Age was determined by counting growth layer groups (GLG) in thin sections of teeth. Jaws were boiled, and teeth were extracted and cleaned. The three largest and least-worn teeth from each beluga were mounted on a small wooden block and thinly sectioned using a Buehler IsoMet low-speed saw equipped with a diamond wafering blade. For each beluga, GLGs in at least two teeth were counted a minimum of two times each by two readers. If needed, additional teeth were counted until an agreed-upon final count for each animal was reached by both readers (Lockyer et al. 2007). Actual age was estimated for animals when the neonatal line of the teeth was visible, while minimum age was estimated for older animals when

the neonatal line had been worn away. In accordance with the findings of Stewart et al. (2006), one GLG was assumed to form annually.

Data analysis

FA data were transformed using the following additive log-ratio approach advocated by Aitchison (1994) for use with compositional data:

$$x_{trans} = \ln(x_i/c_r)$$

where x_{trans} is the transformed FA, x_i is the FA expressed in mass percent of total FA identified, and c_r is the mass percent value of a reference FA. 18:1*n*7 was chosen as the reference FA, as it is commonly quantified in samples processed by most researchers in other laboratories (e.g., Koopman et al. 1996, Walton et al. 2007, Strandberg et al. 2008), and because a preliminary analysis of this dataset indicated that mass % values of 18:1*n*7 were approximately equal in all three layers.

Presence of i-5:0 in multiple tissues

An ANOVA blocked on animal was used to examine the effect of tissue on *i*-5:0 levels. Tukey's HSD tests were used post-hoc to examine between-tissue differences. All tests were done with $\alpha = 0.05$.

Effects of age and sex on i-5:0 levels and stratification

ANCOVA was used to examine the effect of "age" on *i*-5:0 levels. Thorax blubber layers were analyzed separately, and sex was included in the model as a

factor. The analysis was performed on both the full set of observations with GLG measurements (n = 46) and on the reduced set of "known age" animals (n = 25). Regression coefficients were compared to determine if there was any bias due to the inclusion of the right-censored data points (i.e., those with minimum age estimates). In order to make use of the full set of belugas sampled (n = 95), this analysis was repeated using body length as the independent variable. Interaction between sex and the covariate (GLGs or length) was evaluated by including an interaction term in the model for the ANCOVA. If the interaction term was found to be nonsignificant, the ANCOVA was re-run with the interaction term removed. If the interaction term was found to be significant, a "separate slopes" approach was used. If sex was found to be nonsignificant, a simple linear regression was done. All testing was done at $\alpha = 0.05$. Normality was assessed by examining boxplots and QQ-plots prior to the ANCOVA and residual plots afterwards. Homogeneity of variances was assessed using Levene's Test.

In order to make a direct comparison with the work of Koopman et al. (2003), transformed *i*-5:0 data for the outer layer was regressed against body length, and sex was not included as a cofactor.

Stratification of *i*-5:0 (SI.iso5) was explored by computing the absolute difference in *i*-5:0 in the inner and outer blubber layers.

$$SI.iso5 = |iso5_{inner} - iso5_{outer}|$$

ANCOVA was then used to examine the effects of sex and both GLGs and body length (examined separately) on SI.iso5.

In order to make a direct comparison with the work of Koopman (2007), a second stratification index (SI) was computed and then regressed against body length. SI.koopman was calculated using untransformed data for *i*-5:0, 14:0, 14:1*n*5, 16:0, 16:1*n*7, 18:0, 18:1*n*11, 18:1*n*9, 18:1*n*7, 18:2*n*6, 20:1*n*11, 20:1*n*9, 20:5*n*3, 22:1*n*11, 22:5*n*3, 22:6*n*3 as follows:

$$\text{SI.Koopman} = \sum_{i=1}^{16} (|FA_{inner} - FA_{outer}|)$$

for all 16 FA included in the index.

SPSS for Windows (version 11.5; SPSS Inc, 2002) was used for all analyses.

RESULTS

Presence of i-5:0 in multiple tissues

Isovaleric acid levels varied significantly among tissues examined (Figure 3.2). Liver and heart had by far the lowest levels measured, with respective mean values of 0.0017 and 0.017 mass %. Levels of *i*-5:0 in thorax blubber were generally lower than those in tailstock blubber, ranging from a low mean value of 0.63 mass % in inner thorax blubber to a high of 13.57 mass % in outer tailstock blubber. Isovaleric acid was present in the greatest amount in the melon with a mean value of 38.39 mass %.

Effects of age and sex on i-5:0 levels and stratification

Results of the ANCOVA performed on the full GLG dataset (n = 46) were comparable (i.e., regression coefficients were similar) to the results from the known-age animal dataset (n = 25), revealing no bias due to the inclusion of the right censored data points. Thus, the full GLG dataset was used for all subsequent analyses, and only analyses on the full GLG dataset are reported.

There was no significant relationship between *i*-5:0 level and age in the inner layer (ANCOVA, $F_{2,43} = 0.524$, $p = 0.596$). The relationship in the middle layer was significant ($F_{1,44} = 4.089$, $p = 0.0245$): ln(*i*-5:0/18:1n7) = -0.64 + 0.02*GLGs, (R^2 = 0.08), but weaker than the relationship in the outer layer ($F_{1,44} = 76.507$, $p < 0.001$): ln(*i*-5:0/18:1n7) = 0.49 + 0.03*GLGs, ($R^2 = 0.64$) (Figure 3.3). Sex was not a significant factor in either layer.

No significant relationship was found between levels of *i*-5:0 and body length in the inner and middle layers (ANCOVA, inner: $F_{2,92} = 0.344$, $p = 0.710$, middle: $F_{2,92} = 1.165$, $p = 0.336$). The separate slopes approach was employed in the outer layer as the interaction between sex and length was found to be significant in the ANCOVA ($F_{1,91} = 28.770$, $p < 0.001$). Isovaleric acid did increase significantly with body length in the outer layer (Figure 3.4). For females, ln(*i*-5:0/18:1n7) = -2.99 + 1.22*length, and for males, ln(*i*-5:0/18:1n7) = -0.13 + 0.32*length ($R^2 = 0.95$ for both). Isovaleric acid also increased significantly with body length in the outer layer when a simple regression was used, and sex was not included as a cofactor (ln(*i*-5:0/18:1n7) = 2.40 + 0.52*length ($F_{1,93} = 39.509$, $p < 0.001$, $R^2 = 0.30$)).

SI.iso5 was found to increase significantly with both age and length. A single equation describes the relationship with age, SI.iso5 = 2.20 + 0.03*GLGs ($F_{1,44}$ = 17.887, $p < 0.001$, R^2 = 0.29), while separate equations were needed to describe the relationship with length. For females, SI.iso5 = -0.84 + 1.04*length, and for males, SI.iso5 = 1.56 + 0.29*length ($F_{4,91}$ = 891.113, $p < 0.001$, R^2 = 0.98 for both) (Figure 3.5).

SI.koopman was found to have a mean value of 50.90 ± 0.96 SE across all 95 belugas, and ranged from a minimum of 29.76 to a maximum of 72.43. The relationship between SI.koopman and body length was significant ($F_{1,93}$ = 20.586, $p < 0.001$), and is described by SI.koopman = 13.75 + 10.22*length (R^2 = 0.18) (Figure 3.5).

DISCUSSION

Presence of i-5:0 in multiple tissues

The most striking result from the comparison of *i*-5:0 levels among the various tissues on a mass % of FA basis was the near-complete lack of *i*-5:0 in the heart and liver, as compared to its predominance in the melon. This difference is amplified when compared on an absolute basis, calculated by taking into account the tissue lipid content (approximately 2.2 % and 4.2 %, wet-weight, in the heart and liver, respectively, versus 83.1 % averaged across all blubber and melon samples). Whereas lipid content is calculated for the tissue as a whole, mass % values refer only to the FA in the lipid fraction of the tissue (the remaining tissue fractions are

protein, fiber, ash, and carbohydrates (Robbins 1993)). Thus, on an absolute basis the amount of *i*-5:0 in the heart and liver (0.02 and 0.002 in the liver and heart, versus 38.4 in the melon) was even less than that indicated by the comparison of mass % levels. These results agree with the few other studies that have measured *i*-5:0 in a variety of tissues. Using sample analytical techniques nearly identical to those in this study, Koopman et al. (2003) measured a mean *i*-5:0 level of 1.4 ± 0.4 (± SE) mass % in the liver of harbour porpoise (n = 18). Morrii (1980) quantified FA using different analytical techniques, and measured a mass % values of 16.2 in the liver (compared to 97.4 in the melon and 93.9 in outer blubber), in a striped dolphin (n = 1). It should be noted that Morrii's values are expressed as a percentage of only the short chain FA present (i.e., FA of 10 or fewer carbon atoms in length).

The extremely low levels of *i*-5:0 in the liver support the idea that this FA is locally biosynthesized in the blubber and melon tissues, consistent with avoiding the likely toxic effects of *i*-5:0 circulation in the bloodstream during transport from the liver. Further support for this could come from the analysis of blood samples. However, it has been challenging to collect useable blood from belugas taken in the subsistence harvest as it is typically clotted at the time of sampling. An examination of the enzyme activity in the biochemical pathway resulting in the production of *i*-5:0 in the above-studied tissues could be used to test the hypothesis that this FA is indeed locally biosynthesized. Tanaka et al. (1966) outlined the early steps in leucine catabolism and postulated that *i*-5:0 accumulates when a dehydrogenase enzyme fails to catalyze one of the intermediate steps. Setting up the appropriate

assays would allow for the investigation of the possible confounding effects of temperature on enzyme activity. Koopman et al. (2003) and Koopman (2007) noted that a thermal gradient likely exists across the depth of the blubber, and suggested that the activity of the enzyme responsible for *i*-5:0 accumulation is temperature-dependent, which would explain why this FA is found at much higher levels in the outermost blubber layer. Unfortunately, collecting the tissues appropriate for such an analysis from harvested belugas has so far proven prohibitively difficult.

The high levels of *i*-5:0 quantified in melon samples in this study are in agreement with observations made in several other species of odontocetes (e.g., Litchfield & Greenberg 1974, Koopman et al. 2003). As summarized in Litchfield et al. (1975), melon samples from dolphins, porpoises, and monodontids consistently possess high levels of isovalerate acids, including *i*-5:0. These FA are noted to pass sounds at significantly lower velocities than corresponding long-chain FA, thus helping to focus sounds as they pass out of the melon. Litchfield et al. (1975) point out that the lack of comparably high levels of such FA in other odontocete families indicates the likelihood that several biochemical mechanisms for focusing of echolocation "clicks" exist.

Presence and stratification of i-5:0 in blubber

Isovaleric acid in beluga blubber was stratified with depth, and levels in the outer blubber were found to increase more strongly with age than body length, as also observed in harbour porpoise (Koopman et al. 2003). It is not surprising that a

weaker relationship with length was found given that *i*-5:0 is thought to accumulate in the outer blubber layer with age (Koopman et al. 1996), and given that upon reaching physical maturity, body length no longer correlates well with age in either of these cetaceans (Read & Tolley 1997, Suydam 2009).

In contrast to the approach taken by Koopman et al. (2007), whose SI incorporated *i*-5:0 in addition to a number of FA commonly used to estimate diet (Iverson et al. 2004), the stratification index in this chapter evaluated *i*-5:0 separately from dietary FA stratification (Chapter 2). It is remarkable to note that the stratification of *i*-5:0 with age (GLGs) occurred at twice the rate observed in dietary FA. The slope for the regression of SI.mean.ext on GLGs was 0.013, while the slope for the regression of SI.iso5 was 0.027. Furthermore, it is informative to compare the scatterplot of SI.iso5 on body length against the scatterplot of SI.Koopman on body length. While the scales of the SI differ greatly owing to the different number of FA used in their calculation, the same basic pattern is apparent in both scatterplots. Clearly *i*-5:0 is extremely influential when incorporated into SI, and it may be prudent to exclude this FA from such indices depending on the nature of the research question addressed.

Stratification in beluga blubber was previously examined by Koopman (2007), whose measurements ranged from 10.4 to 66.9, with a mean value of 36.3 ± 3.2 (SE) (n = 26). This compares to the range of 29.8 to 72.8, with mean value of 50.9 ± 1.0 (SI.Koopman), measured in this study. While it is possible that the belugas sampled by Koopman were generally younger and therefore less stratified

than those sampled here, it is more likely that the difference in magnitude in the SI calculated in these studies is attributable to the different approaches taken to subdivide blubber into inner and outer depth layers. In this study, blubber was equally divided into thirds. Koopman (2007) divided the blubber into inner and outer layers that were separated by a middle (unanalyzed) layer of approximately 20-30 % of the total blubber depth (see Figure 1 in Koopman et al. 2003). If we set the middle layer equal to 20 %, this would mean that the inner and outer layers of Koopman et al. (2003) would consist of approximately 40 % of the blubber layer, as opposed to the 33.3 % in this study. Given that many FA are observed to follow unidirectional gradients across the depth of the blubber (Chapter 2), including additional "middle" layer material will serve to reduce the differences observed between inner and outer blubber layers. While the overall pattern of increasing stratification with body length was observed in both studies, Koopman (2007) found that this increase was not significant, in contrast to the significant relationship found in this study.

In general, female belugas were found to have greater stratification in *i*-5:0 than male belugas of comparable body length, particularly at body lengths greater than approximately 3 m. However, there was much more overlap between the sexes in SI.iso5 than in the index based solely on dietary FA (SI.mean.ext). This is reasonable given that in possibly selectively mobilizing certain FA (Iverson et al. 1995a), and/or simply greater degrees of mobilization/replenishment with reproductive and lactation costs, lactating females would essentially "increase" the

relative levels of *i*-5:0 in the inner blubber, thereby reducing the degree of stratification across the blubber layer.

Using i-5:0 to estimate age in odontocetes

Accurate age estimation of free-ranging individuals is fundamental to the use of management tools, e.g., potential biological removal (Wade 1998), that are based on population dynamics, as vital rates (e.g., survival, fecundity) are age-specific (e.g., Read & Hohn 1995, Suydam 2009). Additionally, age may be used as a predictor of habitat use (e.g., Danil & Chivers 2006) and response to climate change (Coulson et al. 2001). Belugas, and many other species of odontocetes, are currently aged by counting GLGs in tooth dentine. While the work of Stewart et al. (2006) confirmed that GLGs form annually in belugas, thereby settling the discussion in the literature over the rate of GLG deposition, this method often results in minimum age estimates for older animals as teeth erode and GLGs are lost. Of the 46 belugas that were aged in this study, actual age was determined for only 26 owing to tooth erosion. The aspartic acid racemization technique estimates age based on the ratio of D-aspartic acid to L-aspartic acid in the nucleus of the eye lens. This technique has been successfully used to age bowhead whales (*Balaena mysticetus*) (George et al. 1999) and more recently, narwhals (*Monodon monoceros*) (Garde et al. 2007). In contrast to using GLGs, aspartic acid racemization allows for the estimation of longevity without the downward bias due to tooth erosion. Unfortunately, neither of

these techniques can be easily applied to free-ranging animals as they require the collection of either a tooth or an eyeball.

Using FA to estimate the age of free-ranging cetaceans is appealing because the collection of blubber samples may be done via remote-biopsy in a minimally invasive manner. The potential for such a technique is made obvious by the works of Koopman et al. (1996, 2003) who described a strong relationship between age and *i*-5:0 levels in the outer blubber of harbour porpoise. The relationship with this FA is particularly promising as the metabolism of *i*-5:0 is fairly well understood in odontocetes. As summarized by Koopman et al. (2003), *i*-5:0 does not come from the diet, and is thought to be biosynthesized and sequestered in the outer blubber layer. Recently, Herman et al. (2008) developed the following empirically-derived model to estimate age in killer whales:

$$\text{Age (yr)}_{\text{predicted}} = 50.4*(ai\text{-}15{:}0/15{:}0) + 0.561*(16{:}1n9/i\text{-}16{:}0) - 14.5$$

While this relationship predicted age with good precision ($\sigma = \pm$ 3.8yr), it is concerning that the authors noted that the biochemical mechanisms underlying the apparent relationships between age and the FA in the equation were unknown. Furthermore, the predominant source in the predator for all four FA in the equation were summarized in Iverson et al. (2004) as having "relatively large contributions from both biosynthesis and diet", and in the case of *ai*-15:0, "not well understood". Clearly it would be better to predict age using FA whose occurrence in the predator is well understood, and more importantly, not strongly influenced by diet.

There is great potential for developing the use of *i*-5:0 levels to estimate age in belugas (Figure 3.3 b). In addition to estimating actual ages of older belugas (maximum actual age estimated in this study was 20 years (Table 2.3)), the strong relationship in the outer blubber layer highlights the potential for aging free-ranging animals in a minimally invasive manner. While dietary inferences may be limited if only the outer blubber is collected, age could be estimated, and when combined with genetic data from skin samples (typically also collected when blubber is biopsied) may be used to make inferences about population structure (O'Corry-Crowe et al. 1997). Eyeballs were collected from many of the individuals sampled for FA analysis in this study. Once the aspartic acid racemization technique is calibrated for use in belugas, actual ages will be estimated. This will allow us to increase our sample size so that we may better describe the relationship between age and *i*-5:0, and to then investigate age predication based on levels of this FA in outer blubber samples.

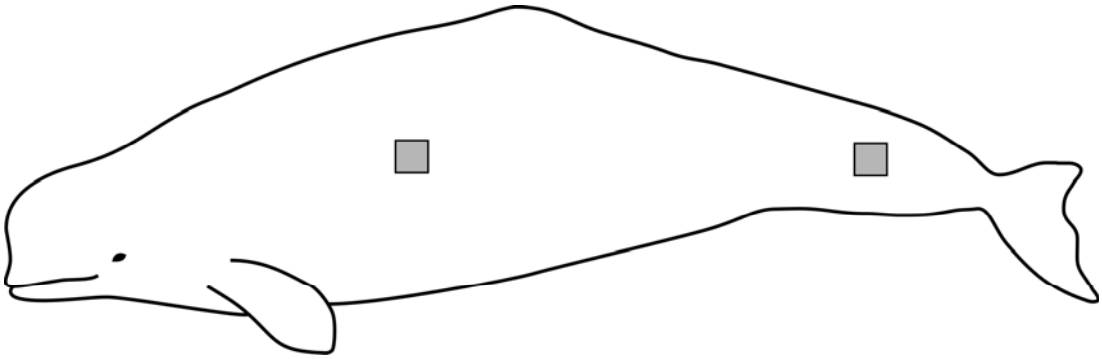

Figure 3.1. Approximate locations of blubber sample collection on the body of the beluga. Full-depth blubber samples (from skin to muscle) were collected from the thorax (n = 95) and tailstock (n = 13).

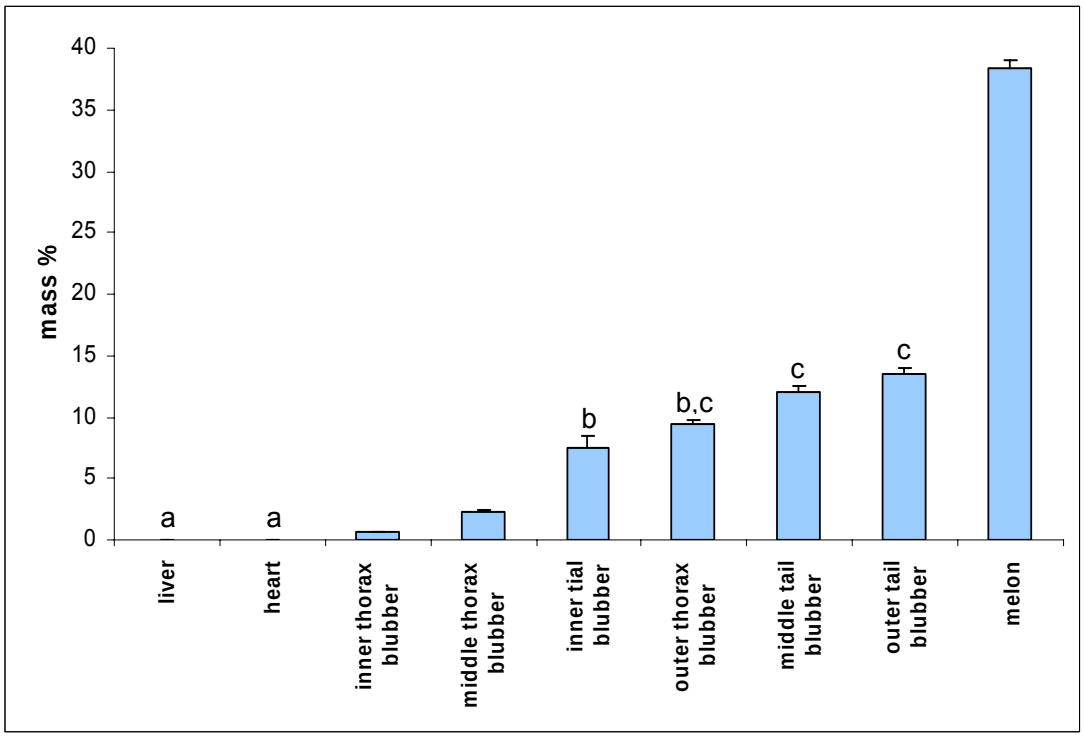

Figure 3.2. Mass % of isovaleric acid in beluga blubber, melon, liver and heart (n = 13). Blubber was collected from the thorax and tailstock, and divided into three depth layers (inner, middle, outer). Bars are means ± SE. Bars with the same lower-case letter were not significantly different (Tukey's HSD, $\alpha = 0.05$). Significance was assessed using transformed data ($x_{trans} = \ln(x_i/c_r)$, where x_{trans} = transformed fatty acid (FA), x_i = mass % of the FA to be transformed, and c_r = mass % of 18:1n7 (reference FA).

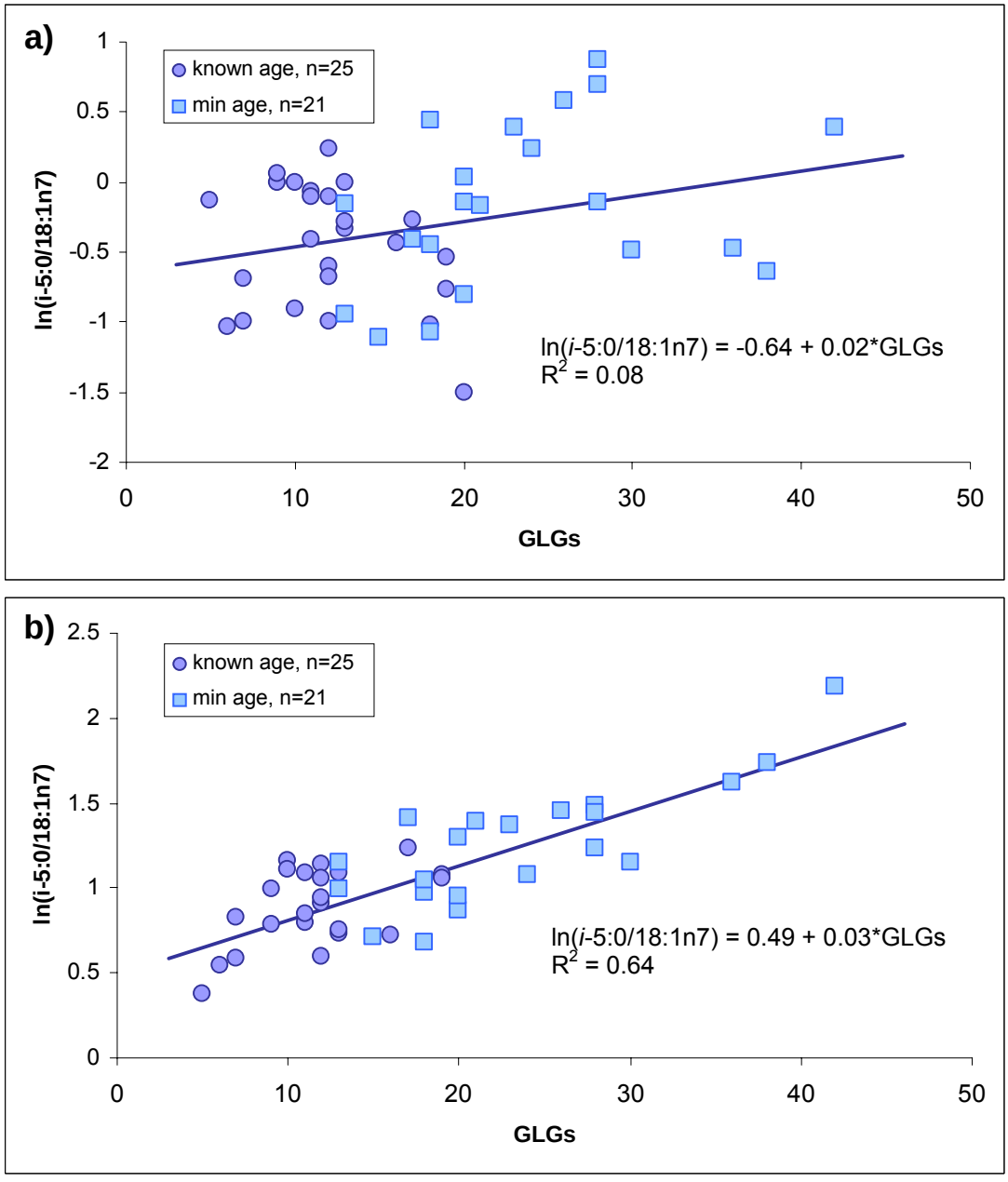

Figure 3.3. a-b. Relationship between isovaleric acid (transformed data) and age (represented by growth layer groups, GLGs, in teeth) in belugas in a) middle and b) outer blubber depth layers. Actual age was estimated for 25 belugas (when neonatal line in tooth was present), and minimum age was estimated for the remaining 21 belugas (neonatal line was absent). A significant relationship existed in both the middle ($F_{1,44} = 4.089$, $p = 0.0245$) and outer ($F_{1,44} = 76.507$, $p < 0.001$) layers. There was no significant relationship between isovaleric acid level and age in the inner layer (ANCOVA, $F_{2,43} = 0.524$, $p = 0.596$).

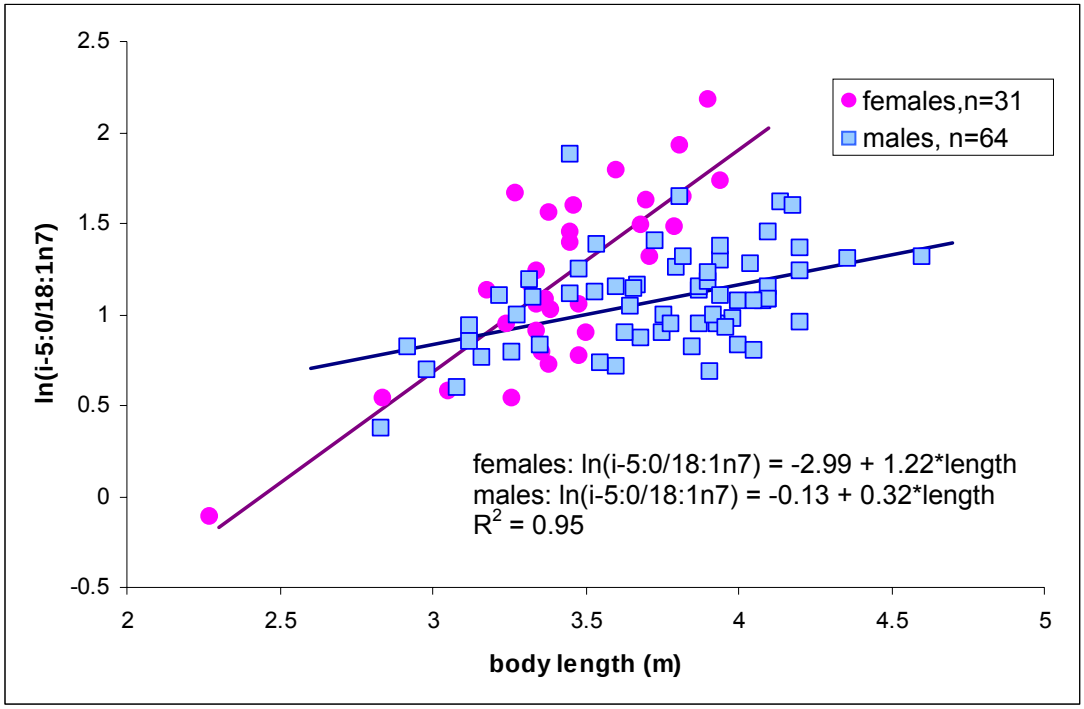

Figure 3.4. Relationship between isovaleric acid (*i*-5:0) (transformed data) and body length in outer depth layer of beluga blubber ($n = 95$). ANCOVA revealed sex to be a significant factor ($F_{1,91} = 28.77$, $p < 0.001$), therefore a separate slopes approach was taken. Significant relationships were found for both sexes. No significant relationship was found between levels of *i*-5:0 and body length in the inner and middle layers (ANCOVA, Inner: $F_{2,92} = 0.344$, $p = 0.710$, Middle: $F_{2,92} = 1.165$, $p = 0.336$).

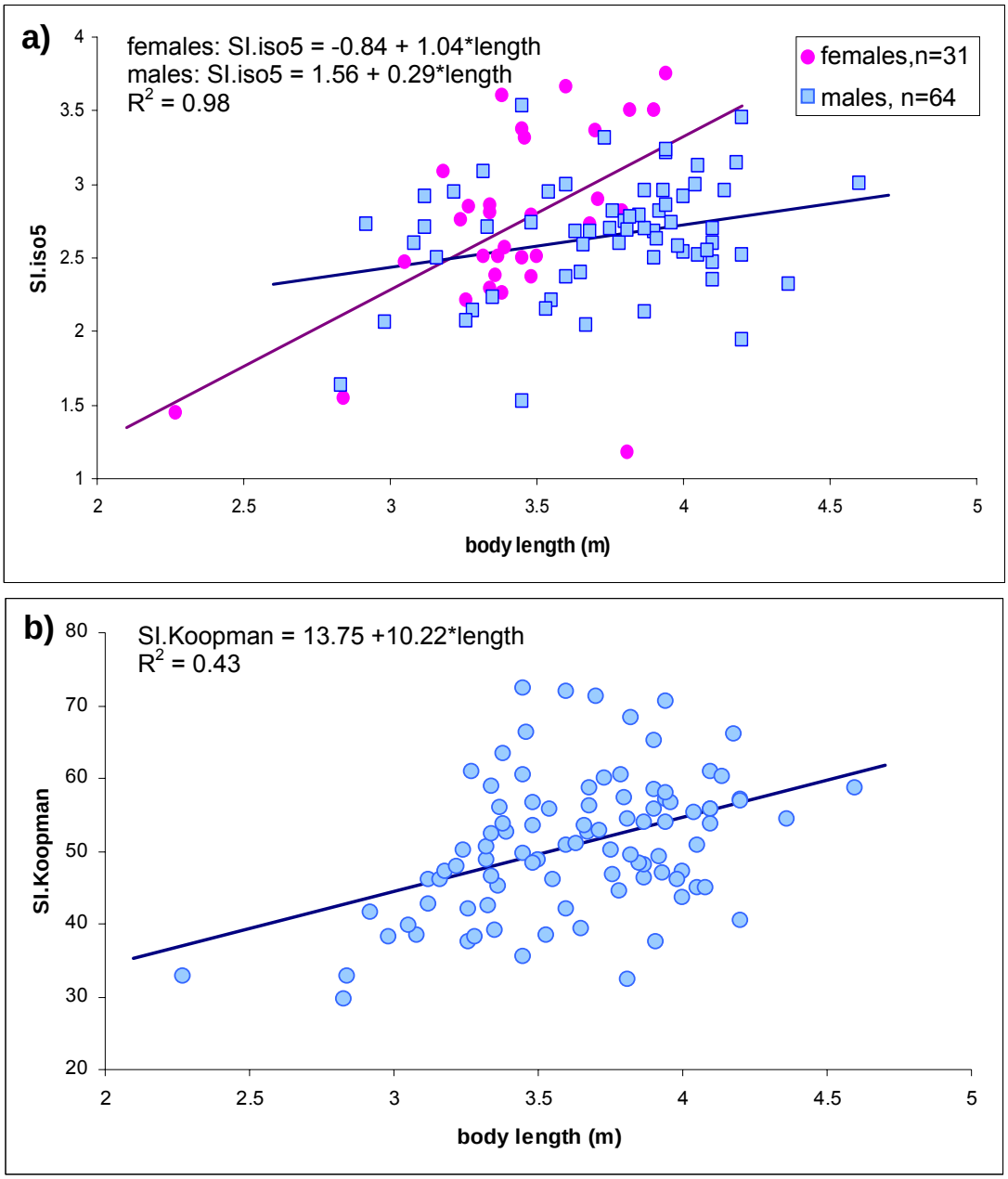

Figure 3.5. a-b. Relationship between fatty acid (FA) stratification and body length in belugas (n = 95). a) SI.iso5 was calculated using transformed data, where SI.iso5 = $|i\text{-}5{:}0_{inner} - i\text{-}5{:}0_{outer}|$. ANCOVA revealed sex to be a significant factor ($F_{1,91}$ = 6.868, p = 0.010), therefore a separate slopes approach was taken. Significant relationships were found for both sexes. b) SI.Koopman was calculated using untransformed data, and sex was not used as a cofactor. SI.Koopman = sum of the absolute value of the differences between 16 FA (Koopman 2007). This relationship was significant ($F_{1,93}$ = 20.586, $p < 0.001$).

Table 3.1. Samples of liver, heart, melon and blubber (thorax and tail) were collected from belugas (n = 13) harvested by subsistence hunters in the village of Point Lay, Alaska in 2006 and 2007. Year of collection is indicated by the last two digits of the animal ID#. Skin colour is used as a proxy for age; young animals are grey, and turn white as they get older. Colour codes: GW = grey-white, W = white.

Sex	Total Length (cm)	Fluke Width (cm)	Skin Colour	Animal ID#
Female	350	80	GW	LDL0506
Female	338	76	GW	LDL2106
Female	346	73	W	LDL1906
Male	355	83	GW	LDL2307
Male	420	100	W	LDL0106
Male	460	97	W	LDL0606
Male	394	86	W	LDL0906
Male	404	87	W	LDL1106
Male	387	85	W	LDL1206
Male	375	81	W	LDL1406
Male	398	100	W	LDL1907
Male	410	99	W	LDL2207
Male	363	85	W	LDL2406

CHAPTER FOUR

THE EFFECT OF NON-DIETARY SOURCES OF FATTY ACID VARIATION ON QUANTITATIVE FATTY ACID SIGNATURE ANALYSIS DIET ESTIMATES FOR AN ARCTIC ODONTOCETE

INTRODUCTION

Quantitative Fatty Acid Signature Analysis (QFASA) is a powerful tool that has been used to estimate the diet of many free-ranging upper-trophic-level predators (e.g., pinnipeds, seabirds) in marine ecosystems (e.g., Iverson et al. 2004, Iverson et al. 2006, Beck et al. 2007, Iverson et al. 2007). However, quantitative diet estimation for cetaceans, and odontocetes in particular, has not yet been attempted given several complications unique to their blubber that require investigation.

First, odontocete blubber is far more vertically stratified than pinniped blubber or non-specialized adipose tissue (e.g., Smith & Worthy 2006, Koopman 2007, Chapters 2 & 3). Regardless of the number of layers into which cetacean blubber has been divided, inner layers (adjacent to muscle) consistently contain higher levels of dietary fatty acids (FA) than outer layers (adjacent to skin). This distribution of FA within the blubber has led to the supposition that the innermost blubber is more metabolically active (Ackman et al. 1975, Lockyer et al. 1984, Koopman et al. 1996), and supports the idea that the depth in the blubber core from which cetacean fat samples are collected must be considered when using FA to make inferences about diet.

While it has recently been specifically recommended that the FA composition of the most metabolically active portion of the blubber layer be used to make dietary inferences (Budge et al. 2006, Iverson 2009b), a number of free-ranging odontocetes have been biopsied remotely, with only the outermost portion of the blubber core collected and available for analysis (e.g., Hooker et al. 2001, Krahn et al. 2007). The consequences of using a less metabolically-active portion of the blubber to estimate dietary relationships are unknown, and could compromise the accuracy of perceptions of diet of odontocetes as reflected in the published literature.

In contrast to the variation in FA composition with depth, FA composition of cetacean blubber is fairly uniform among sites across the thorax (Koopman et al. 1996, Samuel & Worthy 2004, Chapter 2). As such, it is expected that site of sample collection will have negligible impact on diet inferences made using FA.

The second complication, apparently unique to odontocete blubber, is that it may contain significant concentrations of isovaleric acid (*i*-5:0), an unusual branched-chain FA that does not come from diet. Koopman et al. (2003) examined patterns of *i*-5:0 accumulation in the blubber of 30 species of odontocetes and found that dolphins and porpoises in cold waters had relatively high levels of *i*-5:0 in their blubber. Hector's dolphin (*Cephalorhynchus hectori*) had the highest inner blubber level of *i*-5:0 at 18.4 ± 2.8 (wt %, mean ± SE). Isovaleric acid levels in the inner blubber of other species categorized as living in arctic/cold water ranged from 0.8 ± 0.2 wt % in the Long-finned pilot whale (*Globicephala melas*) to 5.6 ± 0.8 wt % in Dall's porpoise (*Phocoenoides dalli*). Mean levels of *i*-5:0 in the inner blubber of

belugas (*Delphinapterus leucas*) have been measured at 0.9 ± 0.1 mass % (*n*=95, Chapter 3) and 2.1 ± 0.6 wt % (n=11, Koopman et al. 2003).

When estimating the diet of a predator using QFASA, non-dietary FA are not used in the model. Thus, the application of QFASA requires a priori deletion from the data set of any FA known to be primarily biosynthesized by the predator rather than originating from dietary sources. Most non-dietary FA are at very low concentrations in marine mammals and have little effect on the subsequently renormalized FA set used in modeling. However, the effects of removing the relatively large amounts of *i*-5:0 quantified in the FA butyl ester (FABE) signature, given that *i*-5:0 does not even appear in the more commonly used FA methyl ester (FAME) signatures available in the published literature (e.g., Iverson et al. 2007, Budge et al. 2008, Tucker et al. 2009), is unknown.

Quantification of short-chained FA, such as *i*-5:0, necessitates the preparation of FABE ($-C_4H_9$) in place of the FAME ($-CH_3$). Short-chain FA, defined as those less than 14 carbons in length, are highly volatile and cannot be accurately measured using FAME. FABE are heavier, less volatile FA derivatives, thus permitting the measurement of short-chain FA (Budge et al. 2006). FA data inputs for QFASA are currently quantified via the use of FAME. It is unknown if ester preparation method affects QFASA output, even when only dietary FA are used in model runs.

Chapter Objectives

I examined the effects of FA variation due to sampling location (blubber depth and body site) and transesterification technique used to process the fat samples (FAME vs. FABE) on quantitative diet estimates for belugas using the QFASA model.

METHODS

QFASA has four requirements, as described in Iverson et al. (2004). First, a predator tissue with a lipid-turnover rate appropriate to the research question at hand (e.g., milk, blood, metabolically active blubber) must be sampled. Second, a "prey library" composed of the species most likely to be consumed by the predator, and with sufficient quantities of individual species to capture within-species variability, must be assembled and analyzed. Third, a set of "calibration coefficients" (CC), used to account for lipid metabolism in the predator, must be available. The set of CC used must be appropriate for the predator under study. For example, it is unlikely that CC empirically determined for seabirds are appropriate for use with belugas. Finally, a statistical model is needed to estimate the composition of the predator's diet. The first three requirements for QFASA serve as the model inputs.

Beluga data

FABE data from beluga blubber samples prepared and described in previous chapters were used in QFASA modeling (Table 4.1). The only additional labwork

required was to prepare FAME for a subset of the blubber samples. FAME were prepared using archived lipid from 2008 inner blubber samples (n = 20) using the Hilditch reagent (0.5 N H_2SO_4 in methanol) according to Budge et al. (2006). Briefly, extracted lipid was heated with methylene chloride (with 0.01 % BHT (v/w)) and Hilditch reagent at 100 °C for one hour. FAME were then extracted into hexane, dried with anhydrous sodium sulfate, and evaporated under nitrogen. Hexane was then added back to FAME to achieve the concentration needed for analysis (50 mg FAME/ml hexane) on the gas chromatograph (GC).

FAME were analyzed in duplicate using temperature programmed gas-liquid chromatography. Gas chromatography methods were identical to those described in Chapter 2, except that the oven programming for FAME was used (Budge et al. 2006). The initial oven temperature of 153 °C was held for 2 min, ramped to 174 °C at 2.3 °C/min, held for 0.2 min, ramped to 210 °C at 2.5 °C/min, and then held for 2 min, producing a total runtime of approximately 32 min. FA were identified, converted to mass percent of total FA, and then named using shorthand nomenclature, as was done in Chapter 2.

Prey data

Species were included in the prey library primarily on the basis of stomach contents data from belugas that winter in the Bering Sea (L. Quakenbush, *unpublished data*). Additional species were included in the prey library when FA data were available for similar, but not identical, species to those identified in

stomach contents (e.g., Rex sole (*Errex zachirus*), Irish Lord sculpin (*Hemilepidotus jordani*)), and when FA data were available for prey items thought likely to be consumed by belugas though not identified in the stomachs analyzed (e.g., capelin (*Mallotus villosus*), Atka mackerel (*Pleurogrammus monopterygius*)). Prey FA data were compiled from a number of sources (S.J. Iverson and A.M. Springer, *unpublished data*, L. Hoberecht, *unpublished data,* Loseto (2007), and S. Wang, *unpublished data*) (Table 4.2).

Calibration coefficients

QFASA utilizes CC, which are calculated from captive feeding studies, and are required for accurate estimates of diet (Iverson et al. 2004). For a given FA, the CC is calculated as the ratio of the quantity present in the predator to the quantity present in the prey consumed (e.g., $CC_{22:1n\text{-}11} = predator_{22:1n11} / prey_{22:1n:11}$). None have yet been estimated for cetaceans. Given the remarkably consistent values determined for CC across a wide range of mammals (Iverson 2009b), I made use of several sets of CC derived for phocid and otariid pinnipeds. Mean values for each species were calculated. The mean across all species means was then used in the model runs (Table 4.3).

QFASA model

The QFASA model was developed by Iverson et al. (2004) and is available as a contributed package (QFASApack) in R (R version 2.8.1, The R Foundation for

Statistical Computing, 2008). This model weights the predator FA signature with the CC to account for the differential, yet predictable, deposition of dietary FA in the predator fat stores. The average FA signatures for each prey type are calculated, and the proportions of the prey types required to produce the weighted predator FA signature are determined. These prey proportions are determined by minimizing the Kulback-Liebler statistical distance between the mixture of prey types in the estimated diet and the weighted predator FA signature.

A reduced version of the "extended" FA subset (Iverson et al. 2004) was used in this modeling exercise (Table 4.3). The extended subset consists of FA that could have only come from ingested prey items, as well as several FA that, while they may be biosynthesized by the predator, are present in quantities influenced by specific prey items ingested. The extended FA subset used here was reduced as only 36 of the 41 FA identified by Iverson et al. (2004) could be reliably identified in this study.

Modeling approach

First, the ability of the QFASA model to discriminate between, and correctly identify, prey species in the library was explored using "prey on prey" (POP) simulations. This simulation exercise is available in QFASApack and functions by treating in turn each species in the library as a predator. When a prey species is designated as the "predator" in a simulation, the number of specimens of that prey species are split in half, with the mean FA signature of the first half of the specimens used as the "predator" signature, and the mean FA signature of the second half of the

specimens used as the prey signature in the library. One thousand such simulations are completed for each prey species in the library. The diet estimates produced for each prey species are then examined to determine other prey species for which they are most commonly mistaken. When a prey species has a very distinct FA signature, the diet estimates produced are composed primarily of that same prey species. For example, in this prey library, the POP simulations showed that Pacific cod (*Gadus macrocephalus*) was identified as itself > 90 % of the time, while slender eelblenny (*Lumpenus fabricii*) was identified as itself 60-69 % of the time, and mistakenly identified as yellowfin sole (*Limanda aspera*) cod 10-19% of the time (Figure 4.1).

POP simulations were used to determine which prey species should be grouped together (e.g., three species of squid were combined to form a single "squid" prey type), and which should be split apart (e.g., saffron cod (*Eleginus gricilis*) was divided into two types based on size). Species were grouped together only when it was ecologically or taxonomically sensible to do so. Prey groups were re-defined, and POP simulations re-run until there was no longer an obvious increase in the success with which prey groups were identified as themselves (the majority of prey groups were identified correctly greater than 80% of the time), and no additional ecologically or taxonomically sensible grouping or splitting of prey species could be justified.

Impact of sampling location on QFASA diet estimates

To compare the effects of sampling from various blubber depths, model runs were completed for each of the three layers for all belugas (n = 95) sampled in Chapter 2. To compare the effects of sampling from various body sites, model runs were completed for the inner layer samples from each of the six sites sampled in Chapter 2 (n = 20) (Table 4.1). ANOVA was used to compare the proportions of the prey species identified among both body sites and blubber depth layers in the diet estimates. The Bonferroni method of modifying the alpha value ($\alpha = 0.05/p$, where p is equal to the number of prey types identified in diet estimates) was used to reduce the increased risk of Type I errors associated with multiple F-tests (Johnson & Wichern 1998). Tukey's HSD post-hoc tests ($\alpha = 0.05$) were used to explore differences in diet estimates with layer (SPSS for Windows, version 11.5; SPSS Inc, 2002).

Impact of lab technique (FAME vs. FABE) on QFASA diet estimates

The FAME and FABE datasets (Table 4.1) were re-normalized to include only the 36 FA used in QFASA model runs. A MANOVA on all 36 FA (arcsine transformed) ($\alpha = 0.05$), followed by ANOVAs ($\alpha = 0.05/36 = 0.0014$) for each individual FA, were used to compare the amounts of the FA quantified using the different laboratory techniques.

To compare the effects of laboratory technique, model runs were completed for both the FAME and FABE datasets (n = 20) (Table 4.1). ANOVA was then used to compare the proportions of the prey species identified in the two model runs.

RESULTS

Impact of sampling location on QFASA diet estimates

Four prey types were identified in the diet estimates for each body site (Figure 4.2). Composition of the modeled diet did not differ significantly between body sites (ANOVA, all $p > 0.85$).

A total of eleven different prey types were identified in the diet estimates for blubber layers. The suite of prey types estimated in the estimates differed with layer (Figure 4.3). Ten species were identified in the inner layer, and of these, four were only identified in the inner layer in very small amounts. Proportions of seven of the 11 species differed significantly with layer (Tukey's HSD, $\alpha = 0.05$) (Figure 4.3).

Impact of lab technique (FAME vs. FABE) on QFASA diet estimates

Lab technique used to prepare the lipid samples for GC analysis resulted in statistically significant differences in quantification for 17 of the 36 FA identified (MANOVA, $p = 0.017$; ANOVA, $p < 0.0014$). In general, FA with 18 and fewer carbon atoms were quantified at higher levels in FABE vs. FAME samples. In contrast, FA with 20 or greater carbon atoms were quantified at higher levels in FAME vs. FABE samples (Figure 4.4). Both ester preparations identified the same

nine prey types in the diet estimates. Proportions of the prey types did not differ significantly with ester preparation (ANOVA, $\alpha = 0.006$) (Figure 4.5).

DISCUSSION

Using QFASA to model odontocete diets

The results clearly demonstrate that blubber samples collected from a range of sites across the thorax of belugas, and by extension most other cetaceans, result in comparable diet estimates using FA data. That the statistically nonsignificant differences in FA composition observed across the body of most cetaceans (e.g., Koopman et al. 1996, Samuel & Worthy 2004, Chapter 2) are also ecologically insignificant will be welcome news for researchers using FA to infer diet in free-ranging animals that are remotely biopsied and cannot be consistently sampled at the same body site (e.g., Hooker et al. 2001, Krahn et al. 2007).

The results also clearly demonstrate that when samples are collected at different depths in blubber with vertically stratified FA, the diet estimates produced will differ in both the number and proportions of prey types identified in the diet. Thus, it is imperative that approximate depth of sample collection be noted and considered when drawing inferences about diet. It is also noteworthy that while many of the prey items identified in small proportions in the inner layer diet estimates were absent in the outer layer estimates (e.g., salmon, rainbow smelt (*Osmerus mordax*), the prey items that predominated the inner estimates (e.g., Arctic cod, Atka mackerel (*Pleurogrammus monopterygius*) and capelin (*Mallotus*

villosus)) were also present in high proportion in the outer estimates. Thus, depending on the nature of the research question being addressed, analyzing the less-metabolically active outer blubber may be adequate.

Finally, it was demonstrated that the esterification technique used to prepare the lipid samples for GC analysis (FAME vs. FABE) did not impact QFASA diet estimates. This is welcome news on two accounts. First, it establishes that for researchers wishing to quantify short-chain FA in the study of non-dietary aspects of lipid metabolism, which can only be done using less volatile FABE, that the same dataset may also be used to study diet (after FA less than 14 carbon atoms in length have been removed). Second, and more biologically interesting, it provides evidence that while some species of odontocetes have high proportions of *i*-5:0 in their blubber (e.g., harbour porpoise (Koopman et al. 1996) and belugas (chapter 3)), this does not appear to impact the overall patterns of dietary FA deposition.

Using QFASA to model beluga diet

This work was a sensitivity analysis, with the objective of evaluating the influence of some non-dietary sources of FA variation unique to cetaceans on QFASA diet estimates. I did not attempt to produce conclusive estimates of beluga diet given that two of the four requirements of QFASA cannot currently be satisfied. Calibration coefficients specific to belugas are still lacking, and the prey library used in the simulations must be expanded to include missing and under-represented potential prey items (e.g., FA data for only Chinook salmon (*Oncorhynchus*

tshawytsch) were available when modeling was done). Regardless of how strongly the diet estimates produced here are qualified, there is undeniable interest in the estimates themselves. While recognizing the limitations of these diet estimates, it is re-assuring to note that the species that dominated all estimates (Arctic cod), has also been identified as the predominant prey item of belugas summering in the eastern Beaufort Sea (Loseto et al. 2009). This bodes well for the future application of QFASA to species less tractable than pinnipeds.

Future work needed

The single biggest problem currently facing this work is the lack of CC specifically derived for belugas or other odontocetes, or indeed for any cetacean species. Calibration coefficients are empirically determined, requiring a captive feeding study and necessitating the cooperation of a zoo or aquarium. In addition to maintaining belugas on a constant diet for the duration of the feeding study, and providing feeding records and samples of the prey items fed during the study, the facility and animal care staff must be willing to subject their display animal(s) to the collection of blubber biopsies. This is especially challenging with belugas given that their skin is smooth and white, and will not conceal the incision that will necessarily be made during the blubber biopsy.

One of the most intriguing aspects of developing CC specific for belugas is the potential to develop separate sets of coefficients that may be applied to samples collected from various depth layers in the blubber. It is conceivable to expect that

CC could be developed to account for the various modes of lipid metabolism that occur with depth, such that samples of outer blubber collected via remote biopsy (e.g., Hooker et al. 2001, Krahn et al. 2007) could be used to provide diet estimates that are as reliable, though likely reflecting foraging patterns over a longer period of time prior to sampling, as those determined using samples of blubber that is metabolically more active.

Captive feeding trials with belugas will be invaluable for determining the rate of FA turnover in the blubber layer(s). This information is necessary for estimating the time period prior to sampling to which QFASA diet inferences may be applied. As well, captive feeding trials will allow us to evaluate how accurately diet is estimated using FA. In the absence of such feeding trials, QFASA diet estimates may be compared with estimates produced using stable isotopes, as was recently done with grey seals (*Halichoerus grypus*) (Tucker et al. 2008).

Finally, additional potential prey items of belugas must be collected and analyzed for inclusion in the prey library. This is a particularly difficult and expensive undertaking given the great distances and remote locations covered by belugas over the course of a year (e.g., Suydam 2009), but it is certainly not impossible. These challenges can be overcome if FA data are shared among researchers (as was done in this study), and if prey collection can be coordinated with fish and invertebrate survey projects already underway.

In conclusion, this study has shown that there is great promise in using the QFASA model to estimate diet in belugas and other cetacean species, and can be used to guide sample collection protocols for these species. While the production of accurate diet estimates remains contingent on satisfying the four requirements of QFASA, this study makes it clear that collecting cetacean blubber and potential prey samples is already a worthwhile endeavour, and that by beginning collections today, a baseline dataset can be developed against which future changes may be compared.

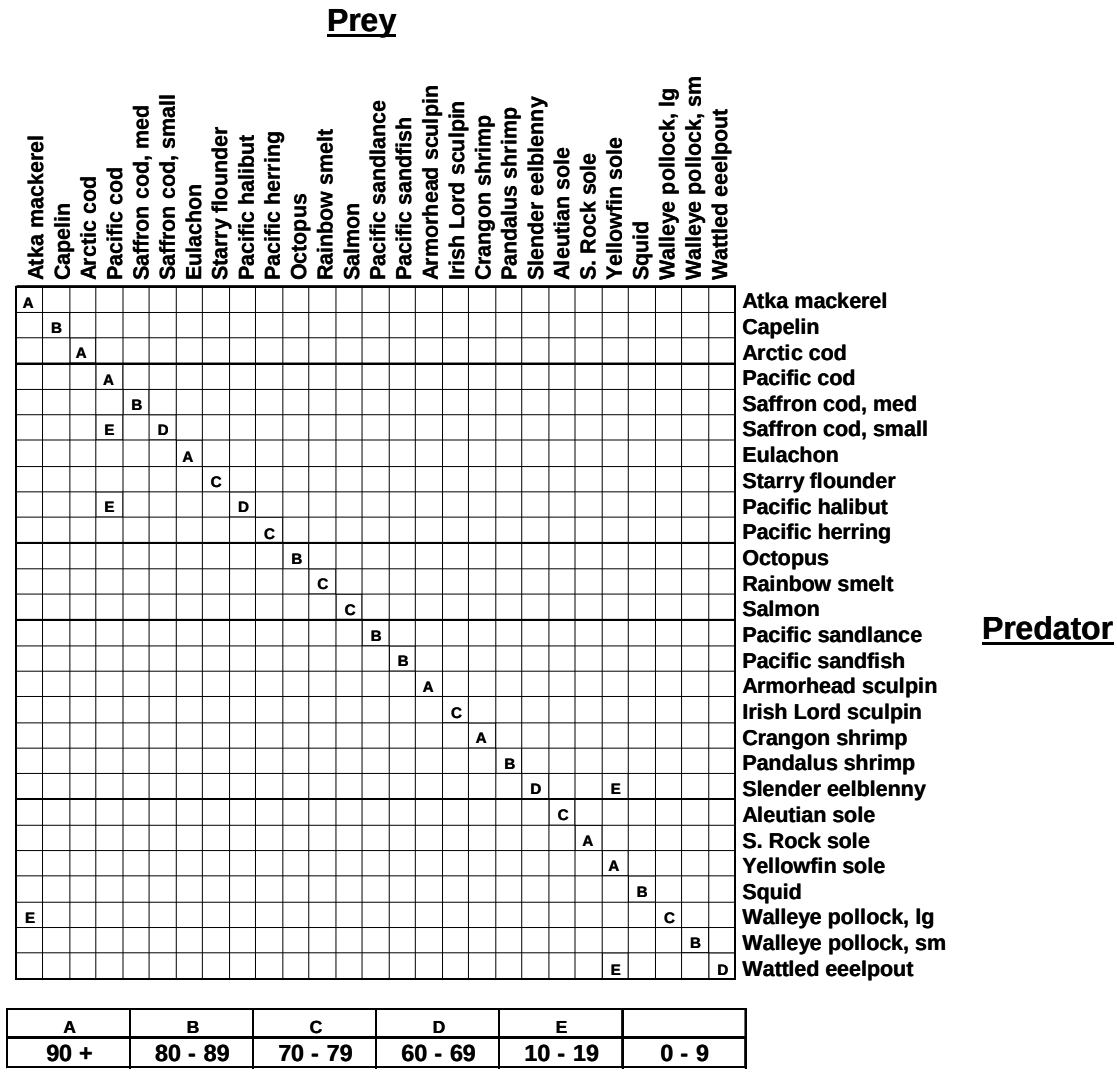

Figure 4.1. Image plot summarizing the results of the "prey on prey" (POP) simulations used to assess the ability of the quantitative fatty acid signature analysis (QFASA) model to correctly identify prey species in the library. Prey species treated as "predators" are listed to the right of the plot. Prey species in the prey library are listed across the top of the plot. Colour of square corresponds with the % of the "predator" estimated as a particular prey species. Prey species that were best identified have the letter "A" running along the diagonal. Note that most of the "white" squares had values equal to zero. Also note that the scale is not continuous as there were no squares with values between 20 and 59%.

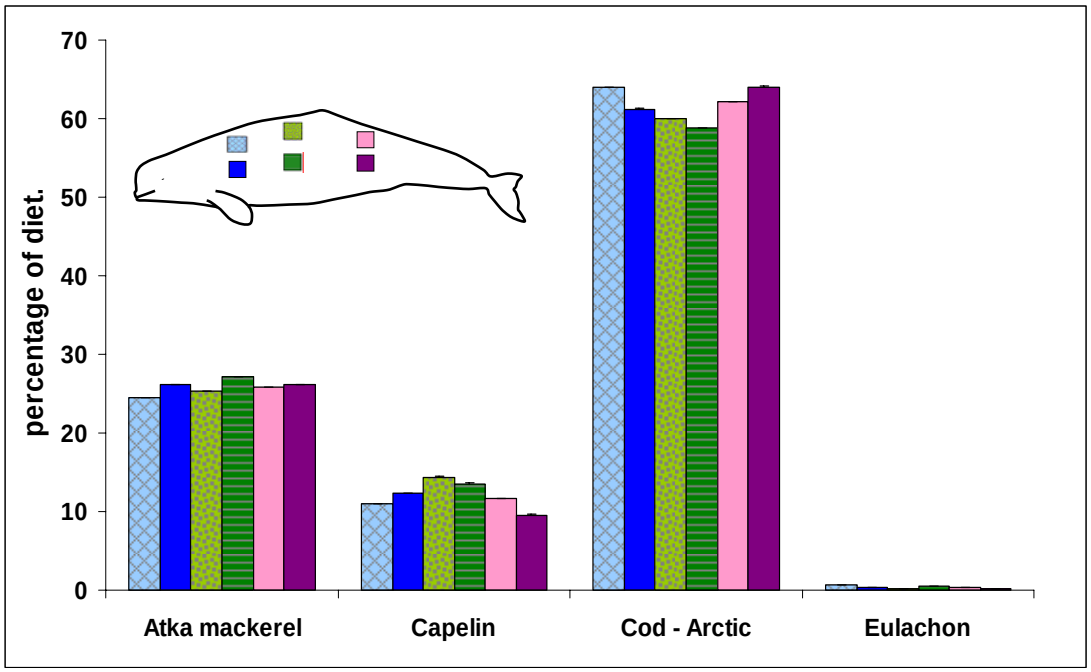

Figure 4.2. Quantitative fatty acid signature analysis (QFASA) diet estimates for six body sites on belugas (n = 20) (inner blubber layer only). Prey types identified in the diet estimates are listed across the x-axis. Bars are mean ± SE (most SE are too small to be seen). Composition of diet did not differ among six body sites (ANOVA, α = 0.05/4 = 0.0125, Bonferroni corrected).

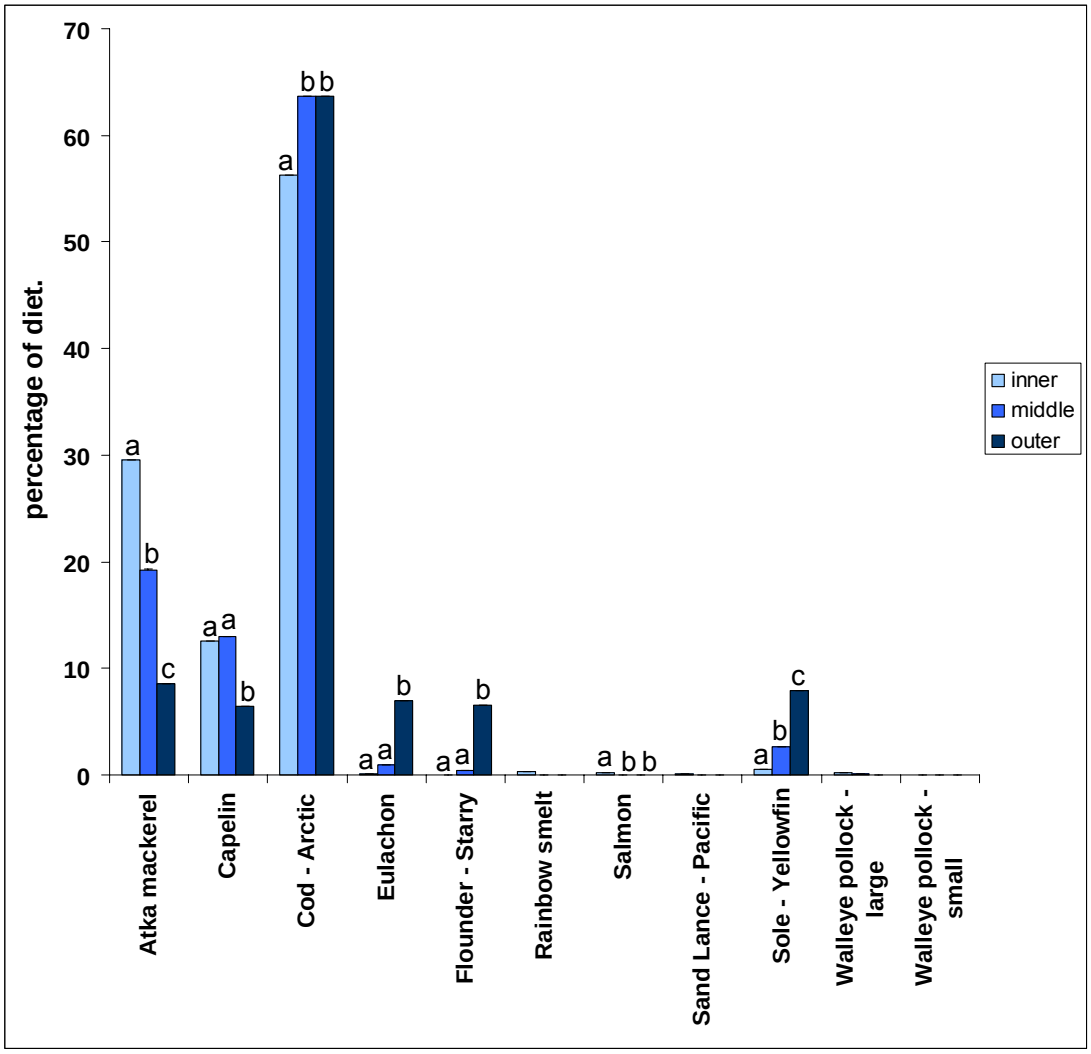

Figure 4.3. Quantitative fatty acid signature analysis (QFASA) diet estimates for three blubber layers ($n = 95$). Prey types identified in the diet estimates are listed across the x-axis. Bars are mean ± SE (most SE are too small to be seen). For prey types where layer was significant (ANOVA, $p < 0.0045$ (Bonferroni corrected)), bars with the same lower-case letter are not significantly different (Tukey's HSD, $\alpha = 0.05$).

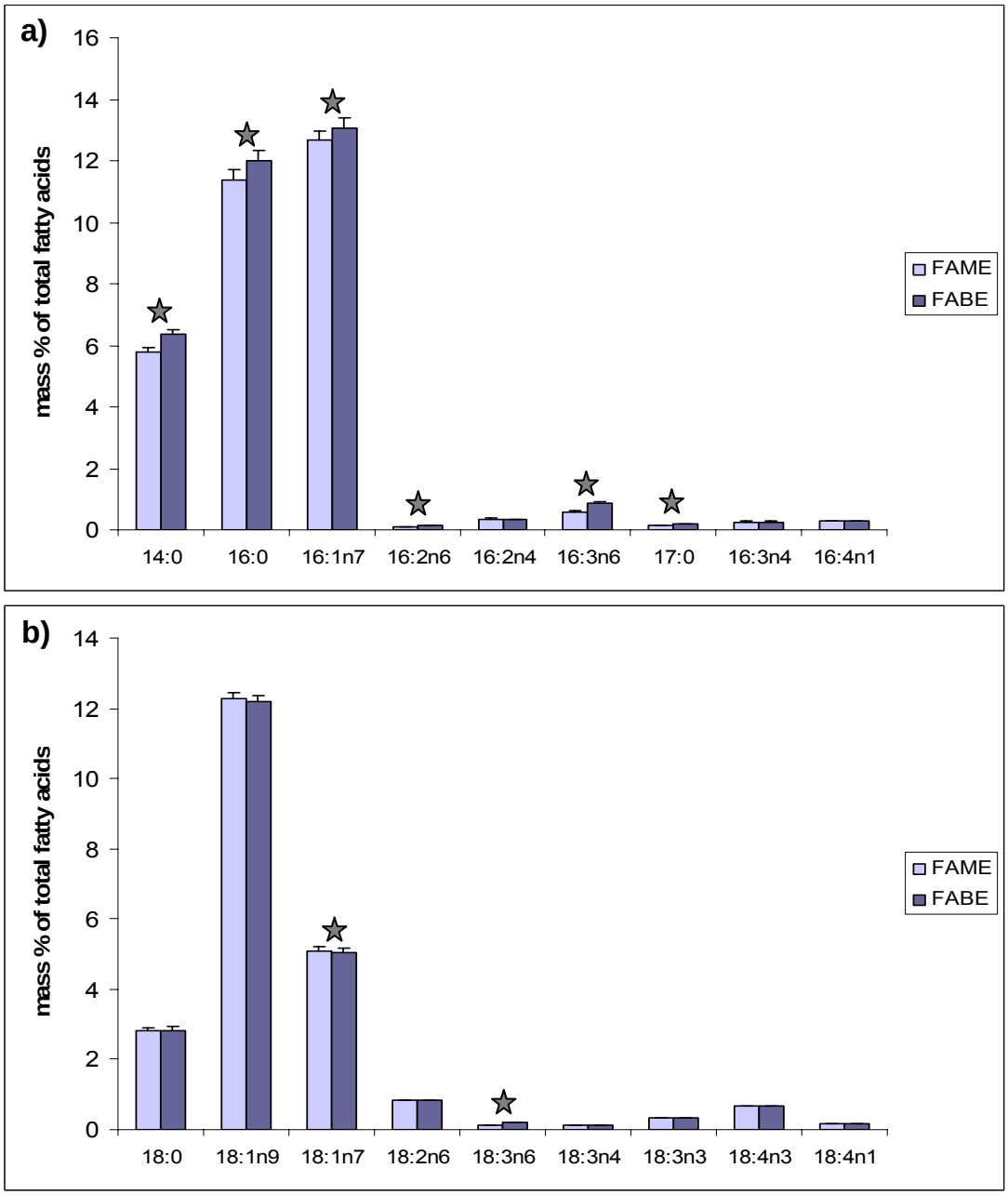

Figure 4.4 a-b. Mass % of "extended" fatty acids (FA) quantified in blubber samples using different lab technique (fatty acid methyl esters (FAME) vs. fatty acid butyl esters (FABE)). Blubber was sampled from the innermost layer from $n = 20$ adult belugas. Bars are means ± SE. Stars indicate FA where lab technique had a significant effect (ANOVA, $p < 0.0014$ (Bonferroni corrected)). Significance was assessed using arcsine transformed data.

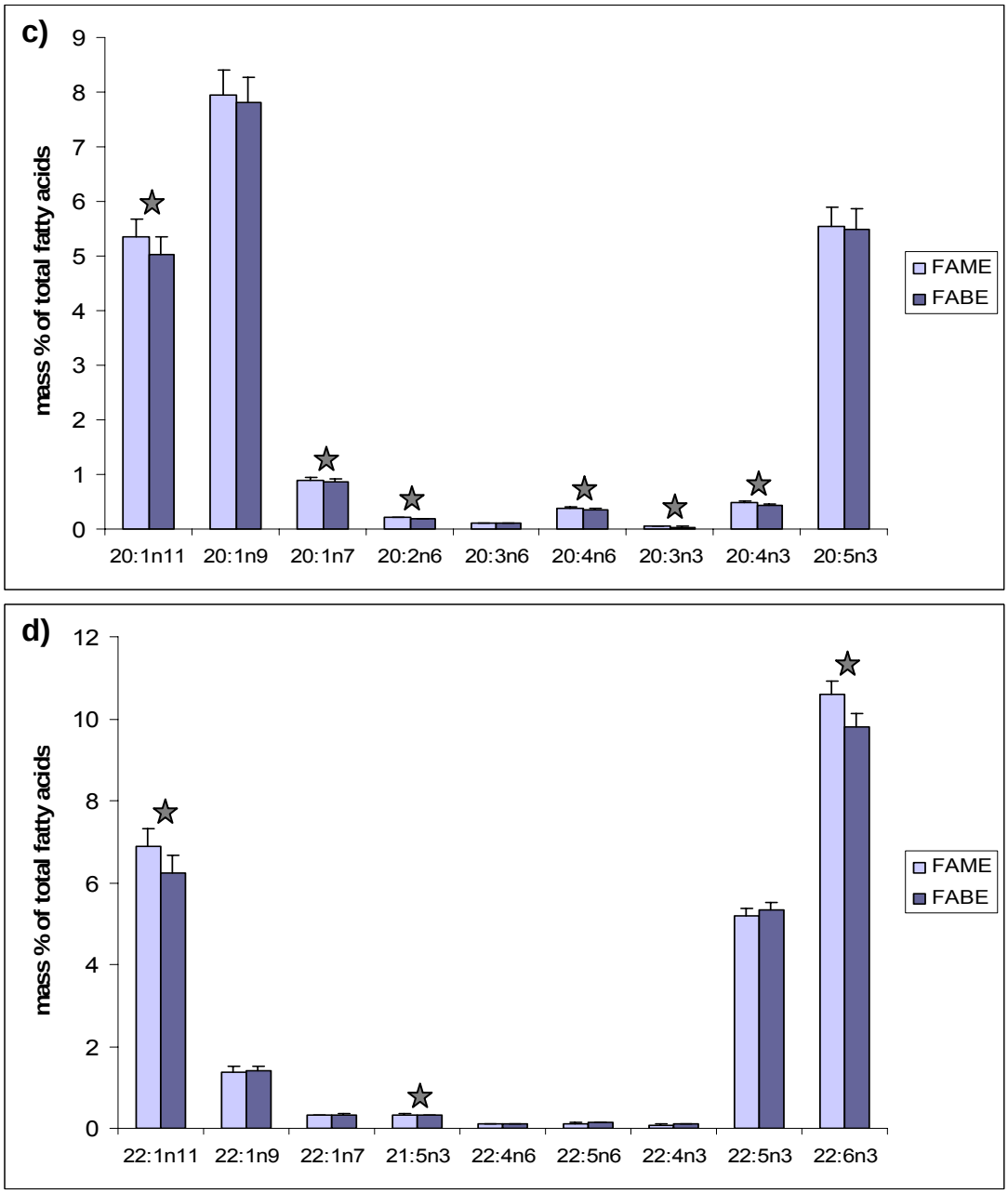

Figure 4.4 c-d. Mass % of "extended" fatty acids (FA) quantified in blubber samples using different lab technique (fatty acid methyl esters (FAME) vs. fatty acid butyl esters (FABE)). Blubber was sampled from the innermost layer from $n = 20$ adult belugas. Bars are means ± SE. Stars indicate FA where lab technique had a significant effect (ANOVA, $p < 0.0014$ (Bonferroni corrected)). Significance was assessed using arcsine transformed data.

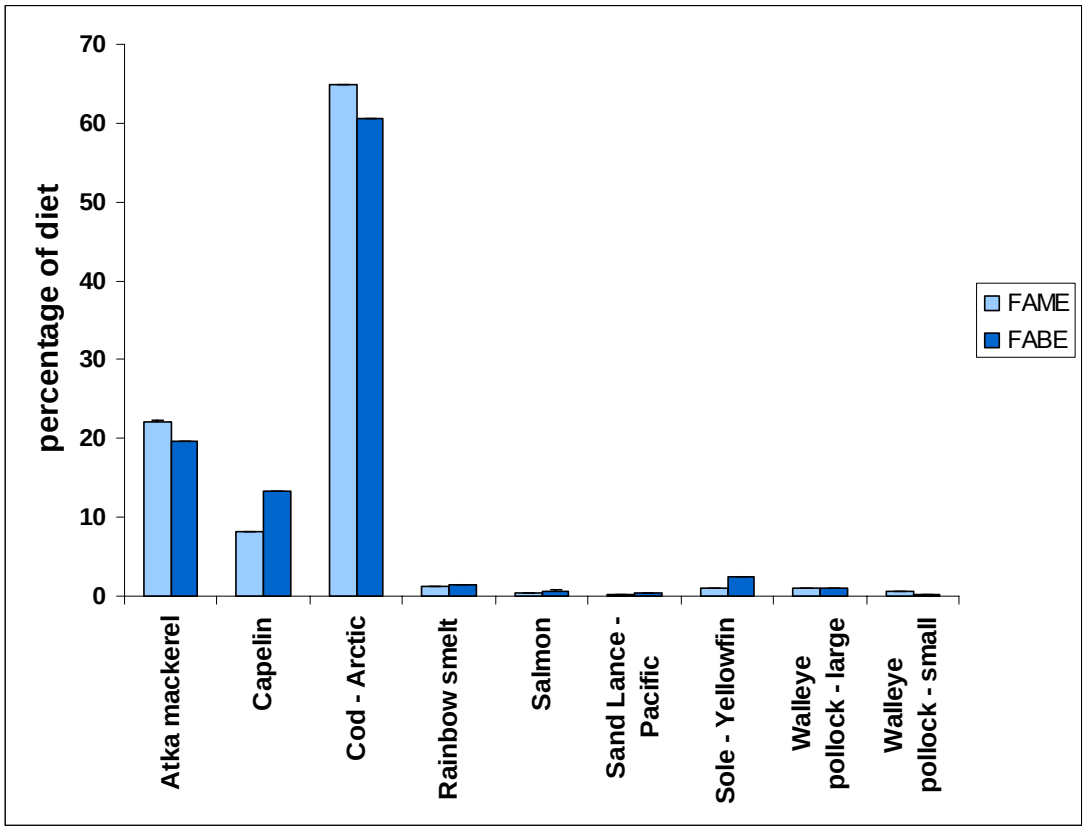

Figure 4.5. Quantitative fatty acid signature analysis (QFASA) diet estimates for fatty acid methyl ester/fatty acid butyl ester (FAME/FABE) comparison (n = 20). Prey types identified in the diet estimates are listed across the x-axis. Bars are mean ± SE (most SE are too small to be seen). Composition of diet did not differ with type of ester preparation (ANOVA, $\alpha = 0.006$, Bonferroni corrected).

Table 4.1. Beluga datasets and sources used to investigate the effects of non-dietary sources of fatty acid (FA) variation on beluga diet estimated using the quantitative fatty acid signature analysis (QFASA) model. The "beluga extended" subset of FA and "average pinniped" calibration coefficients were used in all model runs. Note that FABE = fatty acid butyl ester, and FAME = fatty acid methyl ester.

Factor Under Investigation	**Beluga FA Data Set Used**
Sampling depth (Figure 2.3)	Pt Lay, 2002 & 2005-2008, *n*=95 individuals • FABE data for inner, middle & outer layers body location '4' (Figure 2.2) source: Chapter 2
Body location (Figure 2.2)	Pt Lay, 2002, *n*=20 individuals • FABE data for inner layer 6 body locations source: Chapter 2
Transesterification technique (FABE vs. FAME)	Pt Lay, 2008, *n*=20 individuals • FABE data for inner layer body location '4' source: Chapter 2 • FAME prepared for inner layer body location '4' source: this chapter

Table 4.2. Prey types, collection locations and numbers of specimens in the prey library used to investigate the effects of non-dietary sources of fatty acid (FA) variation on beluga diet estimated using the quantitative FA signature analysis (QFASA) model. Prey types were most often species, however saffron cod was split into two types based on size (small ≤ 80 mm, medium ≥ 150 mm), while "Shrimp-*Pandalus* spp.", "Sole-Aleutian" and "Squid" were groups of similar species.

Prey Type	Scientific Name	Location	Total
Atka mackerel	*Pleurogrammus monopterygius*	Bering Sea[a,b]	46
Capelin	*Mallotus villosus*	Bering Sea[a]	74
Cod - Arctic	*Boreogadus saida*	Beaufort Sea[a,c]	59
		Bering Sea[a]	4
Cod - Pacific	*Gadus macrocephalus*	Bering Sea[a]	97
Cod - Saffron - medium	*Eleginus gricilis*	Bering Sea[a]	17
Cod - Saffron - small	*Eleginus gricilis*	Bering Sea[a]	24
Eulachon	*Thaleichthys pacificus*	Gulf of Alaska[d]	30
Flounder - Starry	*Platichthys stellatus*	Beaufort Sea[c]	14
Halibut - Pacific	*Hippoglossus stenolepis*	Bering Sea[b]	9
Herring - Pacific	*Clupea pallasii*	Bering Sea[a]	172
Octopus	*Enteroctopus dofleini*	Bering Sea[b]	6
Rainbow smelt	*Osmerus mordax*	Bering Sea[a]	49
Salmon	*Oncorhynchus tshawytscha*	Gulf of Alaska[d b]	6
Sand Lance - Pacific	*Ammodytes hexapterus*	Bering Sea[a]	92
Sandfish - Pacific	*Trichodon trichodon*	Bering Sea[a]	11
Sculpin - Armorhead	*Gymnocanthus galeatus*	Bering Sea[b]	5
Sculpin - Irish Lord	*Hemilepidotus jordani*	Bering Sea[b]	10
Shrimp - Crangon	*Crangonidae*	Bering Sea[a]	8
Shrimp - Pandalus spp.	*Pandalus goniurus*	Bering Sea[a]	8
	Pandalus hypsinotus	Bering Sea[a]	2
	Pandalus sp.	Bering Sea[a]	30
Slender eelblenny	*Lumpenus fabricii*	Bering Sea[a]	15
Sole - Aleutian	*Errex zachirus*	Bering Sea[b]	10
	Hippoglossoides elassodon	Bering Sea[b]	13
	Lepidopsetta polyxystra	Bering Sea[b]	13
Sole - Rock - southern	*Lepidopsetta bilineata*	Bering Sea[b]	5
Sole - Yellowfin	*Limanda aspera*	Bering Sea[a]	12
Squid	*Gonatus onyx*	Bering Sea[a,d]	10
	Gonatus madokai	Bering Sea[a,d]	5
	Berryteuthis magister	Bering Sea[b]	5
Walleye pollock - large	*Theragra chalcogramma*	Gulf of Alaska[d b,d]	35
Walleye pollock - small	*Theragra chalcogramma*	Bering Sea[a]	358
Wattled eelpout	*Lycodes palearis*	Bering Sea[a]	14
Grand Total			**1,268**

[a] FA data from S.J. Iverson and A.M. Springer, *unpublished data*
[b] FA data from L. Hoberecht, *unpublished data*
[c] FA data from Loseto (2007)
[d] FA data from S. Wang, *unpublished data*

Table 4.3. Calibration coefficients (CC) for the 36 fatty acids (FA) in the "Extended dietary" subset used to investigate the effects of non-dietary sources of FA variation on beluga diet with the quantitative fatty acid signature analysis (QFASA) model. CCs were calculated as the mean value across all pinniped species[a] for which mean CC have been determined in captive feeding experiments (Iverson 2009b).

Fatty Acid	Calibration Coefficient	Fatty Acid	Calibration Coefficient
14:0	0.91	20:1n11	2.27
16:0	0.70	20:1n9	1.02
16:1n7	1.60	20:1n7	0.90
16:2n6	0.74	20:2n6	1.24
16:2n4	1.00	20:3n6	1.43
16:3n6	0.88	20:4n6	0.97
17:0	0.77	20:3n3	1.12
16:3n4	0.78	20:4n3	1.36
16:4n1	0.59	20:5n3	0.63
18:0	0.79	22:1n11	0.43
18:1n9	1.97	22:1n9	0.55
18:1n7	1.29	22:1n7	0.32
18:2n6	1.43	21:5n3	1.10
18:3n6	1.17	22:4n6	4.51
18:3n4	2.56	22:5n6	1.08
18:3n3	1.15	22:4n3	1.47
18:4n3	0.72	22:5n3	3.05
18:4n1	1.28	22:6n3	0.91

[a] Grey seal (adults and pups) (*Halichoerus grypus*), harbour seal (*Phoca vitulina*), harp seal (*Phoca groenlandica*), Hawaiian monk seal (*Monachus schauinslandi*), and Steller sea lion (*Eumetopias jubatus*).

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