Fatty Acid Metabolism in Marine Carnivores: Implications for Quantitative Estimation of Predator Diets

by

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Abstract

A number of metabolic processes can potentially affect the relationship between dietary and adipose tissue fatty acid (FA) compositions and will influence the use of FA as trophic tracers in predators. The objectives of my thesis were to use juvenile grey seals (*Halichoerus grypus*) and mink (*Mustela vison*) to investigate *de novo* FA synthesis by the liver, the deposition and modification of individual radio-labelled dietary FA, the relationship between dietary and circulating chylomicron FA signatures, and the relationship between dietary and blubber FA signatures in seals fed completely homogenous diets. *In vitro* studies with ^{3}H-labelled acetyl-CoA showed that the primary product of *de novo* FA synthesis in the liver of a marine carnivore was 16:0. *In vivo* studies using labelled FA showed that while some dietary FA are deposited directly, others may be extensively modified. The employment of a dual-label radioisotope tracer technique demonstrated reduced deposition of ^{3}H-22:1n-11, relative to ^{14}C-18:1n-9, in both seals and mink, coupled with the appearance of ^{3}H-labelled shorter monounsaturated products, likely due to peroxisomal chain-shortening in liver. A further study demonstrated that individual dietary FA likely experience differential metabolism in the early assimilation stages leading up to chylomicron formation, thus also affecting incorporation patterns into adipose tissue. Although absolute differences existed, chylomicron FA signatures most resembled diet signatures at peak chylomicron formation and with greater fat intake, likely reflecting the greatest ratio of dietary-triacylglycerol to phospholipid FA. When metabolic aspects were accounted for, chylomicron FA signatures accurately predicted diet composition using a quantitative model. Finally, a series of captive feeding studies with juvenile grey seals demonstrated that even over a relatively brief period of time, FA signatures of a new diet are reflected in predator stores. When differential metabolism of individual FA within the predator was accounted for, blubber FA provided accurate estimations of diet composition using a quantitative model. In addition, while whole blubber provided a longer-term integration of the dietary history of an individual, the inner-most half of the blubber layer provided a view of more recent diet, consistent with expected turnover rates. These studies provide greater insight into the metabolic processes underlying the use of FA to estimate predator diets.

List of Abbreviations

BMR:	Basal metabolic rate
ER:	Endoplasmic reticulum
FA:	Fatty acid(s)
FAME:	Fatty acid methyl ester
FI:	Food intake
GEI:	Gross energy intake
HDL:	High density lipoprotein
HIF:	Heat increment of feeding
HPLC:	High performance liquid chromatography
HSL:	Hormone-sensitive lipase
K-L:	Kulback-Leibler
LDL:	Low density lipoprotein
LPL:	Lipoprotein lipase
MAG:	Monoacylglycerol
ME:	Metabolizable energy
MUFA:	Monounsaturated fatty acid
PL:	Phospholipid
PUFA:	Polyunsaturated fatty acid
QFASA:	Quantitative fatty acid signature analysis
SFA:	Saturated fatty acid
TAG:	Triacylglycerol
TBF:	Total body fat
TBP:	Total body protein
TBW:	Total body water
TLC:	Thin layer chromatography
TWI:	Total water intake
VLDL:	Very low density lipoproteins

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"All things are difficult before they are easy"

I came across this quote while attempting to penetrate the mists of "The Statistical Analysis of Compositional Data" (Aitchison 1986) and felt that it not only summed up my PhD experience but also my life in general. I would like to thank the many people who have been there and helped me through my many transitions from difficult to easy.

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Chapter 1. The Use of Fatty Acids as Indicators of Diet: Metabolic Processes Affecting Adipose Tissue Fatty Acid Signatures

General Introduction

Pinniped Diets

Understanding the diets of free-ranging pinnipeds is critical to evaluating how these animals function within their marine ecosystems. As top predators, pinnipeds may have a large impact on populations of prey species and in turn may be affected by changes in prey availability (Estes 1979, Bowen 1997). For instance, over the last several decades the population size of grey seals (*Halichoerus grypus*) in eastern Canadian waters has increased dramatically (Stobo and Zwanenburg 1990, Zwanenburg and Bowen 1990, Bowen *et al.* 2003). The increased level of grey seal predation on Atlantic cod seen in the 1990s led to the suggestion that grey seal predation could inhibit the recovery of this species on the eastern Scotian Shelf (Mohn and Bowen 1996). In contrast to this situation, populations of harbour seals (*Phoca vitulina*) and Steller sea lions (*Eumetopias jubatus*) in the Bering Sea and the Gulf of Alaska have been declining since the mid-1980s (Pitcher 1990, Loughlin *et al.* 1992, Trites and Larkin 1996) and the nutritional stress hypothesis remains as one of the leading theories to account for the declines (Trites and Donnelly 2003).

Unfortunately, investigations into the diets of pinnipeds are restricted by the difficulties in determining diet over space and time. Direct observation is not a viable option and common methods used in diet studies (stomach content analyses and the

recovery of hard parts from faecal samples) are associated with several well understood but difficult to overcome biases (Bigg and Fawcett 1985, da Silva and Neilson 1985, Jobling and Breiby 1986, Jobling 1987, Dellinger and Trillmich 1988, Bowen 2000, Tollit *et al.* 2003). Perhaps most importantly, these methods can only provide information regarding the animals' most recent intakes. Typically, samples are only collected at near shore haul-out sites and thus do not necessarily represent the offshore diet that these animals rely on during most of the year.

Fatty Acid Signature Analysis

Fatty acid (FA) signature analysis is an alternative method that has been advanced to study the diets of free ranging predators (Iverson 1993, Iverson *et al.* 2004). The most common FA in plants and animals are composed of 14-22 carbon atoms in even-numbered straight chains containing a terminal methyl and a terminal carboxyl. The chains may be saturated or unsaturated with from one to six double bonds. Standard nomenclature identifies individual FA by carbon chain length:number of double bonds and location (n-*x*) of the double bond closest to the terminal methyl group. FA signature analysis is based on the principle that FA in prey species are transferred, in a predictable manner, to lipid storage sites of their predators. Each prey species tends to have a unique pattern of FA, owing to its own life history and dietary habits, which acts like a signature or fingerprint. Because these signatures are conserved through the food chain, they can act as indicators of diet composition (Iverson 1988, Iverson 1993). Marine predators are particularly amenable to the application of FA signature analysis because of the vast

array of different FA commonly present in marine lipids and the presence of large, metabolically active, storage reservoirs of lipid.

There are several advantages associated with FA signature analysis relative to traditional methods of diet analysis. First, because pinnipeds experience periods of fasting and depletion of blubber fat stores (during molting or the breeding season), followed by intensive blubber deposition, FA signature analysis may provide information about the diet that is integrated over weeks or months rather than just giving a snapshot of the animals' most recent intakes. Second, the use of FA does not depend on the presence of hard parts. Thus, all major prey species could theoretically be identified. Third, FA signature analysis can be performed on a very small sample of blubber and provides information for each individual sampled. This constitutes a major advantage considering the ethics of collecting stomachs from sacrificed animals or the usefulness of scats collected from beaches where individuals cannot be identified.

Dietary FA have long been known to influence the FA compositions of their consumers. As long ago as 1935, it was recognized that the FA composition of zooplankton lipids influences the FA composition of the blubber lipids of their baleen whale predators (Klem 1935, Ackman and Eaton 1966). Many researchers have confirmed the direct influence of diet on the FA composition of invertebrates as well as planktivorous and piscivorous fish (Lee *et al.* 1971, Yu *et al.* 1977, Fraser *et al.* 1989, Schwalme 1992, Dos Santos *et al.* 1993, Xu *et al.* 1993, Graeve *et al.* 1994b, Navarro *et al.* 1995, Kirsch *et al.* 1998, Fukuda and Naganuma 2001). Several studies have proceeded to use FA in studying diets in these organisms (Hooper *et al.* 1973, Jeffries 1975, Fraser *et al.* 1989, Klungsoyr *et al.* 1989, Graeve *et al.* 1994a, Kharlamenko *et al.*

1995, St. John and Lund 1996, Graeve *et al.* 1997, Mayzaud *et al.* 1999, Logan *et al.* 2000, Falk-Petersen *et al.* 2001, Fukuda and Naganuma 2001). FA composition of depot lipids reflects dietary FA in several bird species (Moss and Lough 1968, West and Meng 1968, Johnson and West 1973, West and Peyton 1980, Bishop *et al.* 1983, Raclot *et al.* 1998). Dietary FA also influence the FA composition of milk and adipose tissue in terrestrial omnivores (Reidinger *et al.* 1985, Iverson and Oftedal 1992). At higher trophic levels, the influence of fish FA in the diet has been demonstrated in various tissues of several terrestrial and aquatic carnivores (Rouvinen and Kiiskinen 1989, Rouvinen 1992, Rouvinen *et al.* 1992, Colby *et al.* 1993, Pond *et al.* 1995, Kirsch *et al.* 2000). Iverson *et al.* (1995) showed that in rapidly fattening hooded seal (*Cystophora cristata*) pups, dietary FA are so influential that the FA composition of the blubber is almost identical to that of the milk. FA signature analysis has been used, qualitatively, in determining spatial and temporal shifts in diets, both within and between marine mammal species (Iverson 1993, Kakela *et al.* 1993, Smith *et al.* 1996, Iverson *et al.* 1997a,b, Smith *et al.* 1997, Walton *et al.* 2000). It has also been used to infer the dominant prey species of several marine mammals and seabirds (Iverson *et al.* 1997a,b, Raclot *et al.* 1998, Dahl *et al.* 2000).

Quantitative Fatty Acid Signature Analysis (QFASA)

Although studies using FA in a qualitative manner provide strong evidence for the use of FA in elucidating foraging patterns, they do not account for the potential effects of metabolic processes within the predator. As a result of these metabolic processes, the composition of adipose tissue triacylglycerol (TAG, 3 FA esterified to a glycerol

molecule) is never identical to that of the diet. For example, the proportion of monounsaturated FA (MUFA) in predator FA signatures is typically greater than that in the diet (Schwalme 1992, Kirsch *et al.* 1998, Kirsch *et al.* 2000, Summers *et al.* 2000). Also, data for rats show that the ratio of saturated FA (SFA) to polyunsaturated FA (PUFA) is higher in adipose tissue than in the diet (Charnock *et al.* 1985, Roshanai and Sanders 1985, Lhuillery *et al.* 1988). To use FA signature analysis quantitatively, to determine the species composition of diets, a comprehensive understanding of the relationship between dietary and adipose tissue FA compositions is necessary (Iverson *et al.* 2004). This requires a careful evaluation of FA metabolism in predators.

The quantitative assessment of pinniped and other predator diets using FA patterns also requires a statistical model that uses the signatures of probable prey species to compute the mixture of signatures (species and proportions) that most closely resembles that of the predator. Recent work has demonstrated that when the differential metabolism of individual FA within the predator is accounted for, adipose tissue FA compositions can provide accurate quantitative estimates of predator diets (Iverson *et al.* 2004). Calibration coefficients, which are mathematical representations of the relationships between individual dietary and adipose tissue FA, are used in the statistical model of Iverson *et al.* (2004) to weight individual FA as a function of how directly they indicate diet (e.g. whether they originate solely from dietary sources or they are also biosynthesized within the predator). This allows many FA to be included in the model without giving them all the same degree of importance.

Calibration coefficients were developed from long-term feeding studies with the assumption that after such long-term feeding the predator's adipose tissue resembles the

diet as much as it ever would. The calibration coefficient is then calculated as the ratio between adipose tissue and diet levels of each FA. Thus, calibration coefficients provide a composite picture of the influence of differential deposition, modification, utilization and *de novo* synthesis of individual FA on the overall FA composition of blubber. The relative importance of these processes varies with the FA composition and fat content of the diet, ultimately affecting the values of these weighting factors. The direct investigation of the synthesis, modification and deposition of specific FA provides insight into these underlying biochemical processes and can be used to refine calibration coefficients and understand the effects that variation in diet has on them.

Processes of Lipid Digestion, Absorption and Metabolism

Digestion of Dietary Fatty Acids

When a lipid-containing meal is digested by monogastric animals, digestive lipases hydrolyse TAG ester bonds. Lingual and gastric lipases, which begin to work in the stomach, preferentially hydrolyze FA at the *sn*-3 position with the main products being diacylglycerol and non-esterified FA (Cohen *et al.* 1971, Paltauf *et al.* 1974, Hamosh *et al.* 1981, Iverson *et al.* 1992). Liao *et al.* (1984) showed that, *in vitro*, both lingual and gastric lipases preferentially hydrolyze TAG containing FA with fewer than 12 carbons over those containing longer-chain FA. The diets of most carnivores, however, are usually devoid of short- and medium-chain FA, raising questions regarding the importance of these lipases to dietary lipid absorption in these animals. Iverson *et al.* (1992) showed that, *in vivo*, gastric lipase from three species of seal pups readily

hydrolyses milk TAG containing long-chain FA. In fact, the FA that were preferentially released were the very long-chain n-3 PUFA, likely reflecting their prevalence in the *sn*-1,3 positions of the TAG.

Pancreatic lipase also has a specificity for releasing FA from the *sn*-1- and 3-positions of the TAG, leaving mainly non-esterified FA and *sn*-2 monoacylglycerols (MAG) (Mattson and Volpenhein 1964). Pancreatic lipase appears to have a reduced ability to hydrolyse TAG ester bonds containing long-chain n-3 PUFA (e.g., 20:5n-3 and 22:6n-3), possibly due to a steric hindrance created by the acyl chain (Bottino *et al.*1967) but this has been contested (Chernenko *et al.* 1989). This, however, is not an issue for most aquatic predators because the n-3 PUFA in fish lipids are primarily esterified at the *sn*-2 position, allowing them to be readily absorbed as 2-MAG upon pancreatic lipase action (Brockerhoff *et al.* 1963, Brockerhoff and Hoyle 1963, Brockerhoff *et al.* 1968, Litchfield 1968, Chernenko *et al.* 1989, Herzberg *et al.* 1992).

In the majority of fish species studied by Brockerhoff (1966) the SFA (14:0 and 16:0) were present at the *sn*-2 position in greater than random proportions and MUFA were consistently under-represented at the *sn*-2 position. This is to the advantage of their predators as de Fouw *et al.* (1994) and Brink *et al.* (1995) found that, *in vivo*, SFA are more readily absorbed in rat intestine when they are present at the *sn*-2 position and can be taken in as 2-MAG rather than non-esterified FA. In contrast, once hydrolyzed from dietary TAG, SFA are absorbed more slowly than unsaturated FA (Ockner *et al.* 1972, Bee *et al.* 1995). For example, Ockner *et al.* (1972) found that, *in vivo*, 16:0 required a greater length of intestine for its absorption than did 18:2n-6. Several reasons for this have been identified. SFA have higher melting points than unsaturated FA, generally

above body temperature, which may restrict their incorporation into the liquid phase (de Fouw *et al.* 1994, Brink *et al.* 1995). Also, FA soaps (Ca^{2+} or Mg^{2+} salts of long-chain FA), which are much less soluble in the intestinal lumen than the corresponding non-esterified FA, can be formed. Since SFA take longer to be absorbed they are more likely to form FA soaps. In addition, Ca^{2+} and Mg^{2+} soaps of SFA are less soluble in the intestinal fluid than those of unsaturated FA, thus being less absorbed (Jandacek 1991).

Absorption of Dietary Fatty Acids

The absorption of long-chain FA from the intestinal lumen involves several steps: diffusion through the unstirred water layer, passage through the plasma membrane, cytosolic activation and esterification, lipoprotein formation and exocytosis.

The layer of water over the microvilli of enterocytes is not in equilibrium with the bulk aqueous phase of the intestinal lumen and is therefore known as the unstirred water layer. This layer constitutes a diffusion barrier and the hydrophobic long-chain FA can only gain access to the microvilli of enterocytes as mixed micelles in association with bile salts. The micellar solubilization increases the aqueous concentration of FA and MAG 100 to 1000 fold, thus enhancing the number of molecules that are available for uptake by the enterocytes. The acidic microclimate at the surface of the brush border then reduces the solubility of long-chain FA in micelles and promotes their release (Tso 1994).

The process by which FA are taken into cells is not fully understood. The amphipathic nature of FA has lead to a debate regarding whether FA cross cell membranes by passive diffusion or by protein-mediated transport. Contradictory reviews

(Hamilton 1998 vs. Abumrad *et al.* 1998) have been published but evidence from various sources strongly supports the concept that cellular uptake of FA is at least partially due to protein-mediated transport. With respect to the intestinal absorption of FA from the lumen, two membrane lipid-binding molecules have been identified: plasma membrane FA-binding protein and FA translocase (Besnard *et al.* 1996). The localization of both these proteins and the finding that FA translocase expression is regulated by chronic high-fat diets and hypolipidemic drugs (Poirier *et al.* 1996) are consistent with their proposed roles in FA membrane transport. Glatz and van der Vusse (1996) point out that the functions of non-esterified FA trapping and membrane translocation do not necessarily need to be carried out by the same protein. Plasma membrane FA-binding protein is a peripheral membrane protein (Stump *et al.* 1993) that is more likely to act as a FA acceptor, whereas FA translocase is a transmembrane protein that could function in transmembrane FA movement (Schaffer and Lodish 1995).

Once in the cell, long-chain FA bind to cytosolic FA-binding proteins, which are believed to aid in desorption of long-chain FA from the plasma membrane and their diffusion through the cytosol (Glatz and van der Vusse 1996). Several reports have shown the co-expression of FA translocase and cytosolic FA-binding proteins suggesting a co-ordinated functioning of these proteins in cellular FA handling. For example, FA translocase shows similar patterns of expression and regulation to that of adipocyte lipid binding protein (Amri *et al.* 1995), heart FA-binding protein expressed in heart and skeletal muscle and mammary gland (Van Nieuwenhoven *et al.* 1995, Spitzberg *et al.* 1995), and liver FA-binding protein and intestinal FA-binding protein expressed in the gut (Poirier *et al.* 1996).

Several studies have been carried out in an attempt to understand the mechanism of transfer of FA from FA-binding proteins to phospholipid (PL) membranes. Storch and Bass (1990) found that the mechanism of transfer of long-chain FA from liver FA-binding protein to membranes is most likely aqueous phase diffusion. The rate of transfer is influenced by both the structure of the non-esterified FA ligand (unsaturated FA transfer more rapidly than SFA and increases in chain length decrease transfer rate) and the properties of the aqueous phase (Kim and Storch 1992). Hsu and Storch (1996) suggest that long-chain FA transfer from intestinal FA-binding protein to membranes occurs via direct collision of the protein and the PL bilayer, implying a more direct involvement in the targeting of long-chain FA to subcellular membrane sites. Dissociation constants for rat intestinal FA-binding protein, with different FA, varied by 80-fold (Richieri *et al.* 1994). The dissociation constants for intestinal FA-binding protein increased and affinity, therefore, decreased with increasing aqueous solubility of the FA. Of the FA tested, intestinal FA-binding protein had the greatest affinity for 18:0 and 18:1 and the lowest affinity for 18:3n-3. If intestinal FA-binding protein truly is responsible for targeting FA to sites of utilization, this variation in affinity for different FA could translate into FA such as 18:0 and 18:1 being preferentially incorporated into chylomicron-TAG (Lowe *et al.* 1987).

Incorporation of Dietary Fatty Acids into Chylomicrons

Chylomicrons are particles, synthesised in the small intestine, which transport fat and fat-soluble vitamins in the aqueous environment of the blood. They consist of a core of TAG and cholesterol esters and a monolayer of PL, cholesterol and apolipoproteins.

Chylomicron formation begins in the smooth endoplasmic reticulum (ER) and nascent chylomicrons accumulate in the Golgi apparatus (Hay *et al.* 1986). Nascent chylomicrons undergo apolipoprotein glycosylation prior to secretion (Magun *et al.* 1985). Secretory vesicles form and are directed, by a process involving microtubules (Reaven and Reaven 1977), toward the basal-lateral membrane of the enterocyte where exocytosis occurs (Sabesin and Frase 1977, Hay *et al.* 1986).

The first step in the formation of chylomicron-TAG is the activation of non-esterified FA to fatty acyl-CoA, catalysed by acyl-CoA synthetase. Acyl-CoA synthetase is a membrane-bound protein present on the ER, peroxisomes and mitochondria (Krisans *et al.* 1980). The acyl-CoA-binding protein binds fatty acyl-CoA with high affinity and is highly expressed in the liver, kidney and small intestine (Knudsen 1990). Rasmussen *et al.* (1994) showed that, *in vitro*, acyl-CoA-binding protein is capable of transporting acyl-CoA to mitochondria or ER for β-oxidation or TAG synthesis, respectively.

The main fate of dietary FA in the enterocyte is re-esterification in the ER. Two distinct pathways for the synthesis of TAG exist in the enterocyte. During the postprandial period, the 2-MAG pathway is the most important, but the β-glycerophosphate pathway becomes dominant during the interprandial period and fasting (Besnard *et al.* 1996). The enzymes of the former pathway are located in the cytoplasmic face of the smooth ER whereas those of the latter pathway reside in the rough ER (for review, see Tso 1994 and Besnard 1996). Newly synthesized TAG must therefore be transported into the cisternae of the ER and Lin *et al.* (1994) have identified microsomal TAG transfer protein as the membrane protein that accomplishes this.

The *in vivo* partitioning of absorbed FA between newly synthesized PL and TAG appears to vary for individual FA, with 20:4n-6 showing the highest incorporation into PL synthesised in rat small intestine (Nilsson *et al.* 1987, Nilsson and Melin 1988 as cited in Nilsson *et al.* 1992). Perez *et al.* (1999) showed that in enterocytes isolated from rainbow trout (*Oncorhynchus mykiss*), the amounts of ^{14}C-labelled 16:0, 20:4n-6, 20:5n-3 and 22:6n-3 recovered in the polar lipid fraction were higher than those of ^{14}C-labelled C_{18} FA. Conversely, esterification rates of 18:1n-9, 18:2n-6 and 18:3n-3 into TAG were higher than those of 20:4n-6, 20:5n-3 and 22:6n-3. If partitioning of dietary FA between PL and TAG occurs to any extent in the enterocytes of marine carnivores such as pinnipeds, it could affect the relationship between dietary and blubber FA compositions. Those FA esterified in TAG, and eventually incorporated into chylomicrons, would be more readily incorporated into adipose TAG than those esterified in chylomicron PL. To the best of my knowledge, studies of this nature have not been carried out in marine carnivores so the role of differential partitioning of FA between enterocyte TAG and PL in these animals remains unknown.

Dietary Fatty Acid Modification in the Small Intestine and Chylomicron Incorporation

The possibility that the intestinal mucosa is not just the initial site of FA absorption but also the body's first site of chain elongation and desaturation has been put forth by several investigators. Rabinowitz and Myerson (1994) found that when rats were fed doses of either [1-^{14}C]18:1n-9, [1-^{14}C]16:0 or [1-^{14}C]18:0, proportions of labelled FA recovered from the thoracic ducts were similar regardless of which labelled FA had been administered. The authors calculated that 20% of the administered FA had

been inter-converted over the period of the experiment. Bonanome and Grundy (1988) hypothesized that the low cholesterolemic effect of 18:0, in humans, was due to an extensive conversion of 18:0 to 18:1n-9 in the intestine, as evidenced by a rise in the 18:1n-9 content of plasma TAG after feeding an 18:0-enriched diet. Others have also found that 18:1n-9 is over-represented in human chylomicron TAG relative to the diet (Wood *et al.* 1964, Sakr *et al.* 1997).

The results of Bergstedt *et al.* (1990) contradict the finding of Bonanome and Grundy (1988) by showing only a modest increase in 18:1n-9 output in lymph after rats were fed a tristearate (18:0) emulsion. Bergstedt *et al.* (1990) provide an alternate explanation for the over-representation of 18:1n-9 in chylomicron relative to the diet. They demonstrated that the degree of unsaturation of individual FA may affect their packaging into chylomicrons. When rats were administered a tristearate (18:0) lipid emulsion, the lipid absorbed by the intestine was transported into lymph less efficiently than when a trioleate (18:1) lipid emulsion was used. The re-esterification of MAG and non-esterified FA to form TAG was found to be equally efficient with both lipid emulsions but the packaging of lipid into chylomicrons was significantly less efficient with the tristearate emulsion.

Christiansen *et al.* (1986) have demonstrated the existence of a chain elongation enzyme activity, with a preference for SFA and very long-chain MUFA, in microsomal fractions of rat small intestine. This finding was confirmed by Thomassen *et al.* (1990). Garg *et al.* (1988) show that rat small intestine also possesses Δ9- and Δ6-desaturase activity and is capable of converting 16:0 to 16:1n-7 and 18:2n-6 into 18:3n-6. Presumably this Δ9-desaturase activity could also be responsible for the apparent

conversion of 18:0 to 18:1n-9 observed by Wood *et al.* (1964), Bonanome and Grundy (1988) and Sakr *et al.* (1997). The findings of Caselli *et al.* (1993) confirm the ability of rat small intestine, under *in vivo* conditions, to convert dietary 18:2n-6 into 20:4n-6. Chen and Nilsson (1994) show that, *in vitro*, rat small intestine is also capable of the desaturation-elongation of [C^{14}]18:3n-3 to [C^{14}]20:5n-3, however, *in vivo* only a small amount of the dietary [C^{14}]18:3n-3 was metabolized. The authors suggest that this may be due to the rapid acylation of [C^{14}]18:3n-3 into PL and TAG during its absorption and incorporation into chylomicrons.

These FA inter-conversions within the small intestine and subsequent incorporation into chylomicron TAG may be important to the correlation of predator dietary and adipose tissue FA. Summers *et al.* (2000) suggest that, *in vivo*, it is the early metabolic processing of FA that determines their deposition in the adipose tissue of humans. They showed that there are differences in the extent to which FA in the diet are stored in the adipose tissue (n-3 PUFA < SFA < n-6 PUFA < MUFA with 18:1n-9 being stored in greatest amounts) and that the stage at which differential metabolism of FA occurs is their intestinal absorption and incorporation into plasma chylomicron TAG. Similar studies have not been performed on carnivores that are adapted to diets high in n-3 and n-6 PUFA. It is possible that efficiencies of incorporation of these long-chain PUFA are much greater in marine species, as is suggested from studies of neonatal lipid digestion and deposition (Iverson *et al.* 1992, Iverson *et al.* 1995, Layton 1998).

Hydrolysis of Chylomicron TAG by Lipoprotein Lipase

After the synthesis and secretion of chylomicron TAG, a key event in its metabolic processing is hydrolysis by lipoprotein lipase (LPL) and subsequent release of

FA. LPL is expressed in a variety of tissues including adipose tissue, skeletal muscle, mammary tissue, and myocardium. LPL is synthesised within the cell and then exported to its site of action, the luminal surface of the capillary endothelium (Eckel 1989). LPL action is specific for the *sn*-1,3 ester linkages in an acylglycerol, and, *in vitro*, 2-MAG can accumulate during LPL action (Nilsson-Ehle *et al.* 1973, Scow and Olivecrona 1977). Fielding *et al.* (1993, 1995a,b) and Summers *et al.* (1999), however, have shown that, *in vivo*, no MAG is released into venous plasma during situations of high LPL action, suggesting either complete hydrolysis of TAG or tissue uptake of MAG. Summers *et al.* (1999) present results consistent with the hypothesis that, *in vivo*, lipoprotein particles are anchored to LPL for a period of time sufficient for the isomerisation of 2-MAG into 1-MAG and thus complete hydrolysis.

It has been suggested that the initial hydrolysis of FA from chylomicron-TAG may be FA-specific but this is somewhat controversial (Summers *et al.* 2000). Nilsson and Landin (1988) showed that when chylomicrons containing [^{3}H]20:4n-6 and [^{14}C]18:2n-6 were intravenously injected, [^{3}H]20:4n-6 was cleared from the blood more slowly and more appeared in the liver and less in the adipose tissue relative to [^{14}C]18:2n-6. This corresponds well with *in vitro* findings that showed 20:5n-3, 20:4n-6 and 22:6n-3 to be hydrolysed by LPL at a slower rate than the C_{14}-C_{18} acid esters (Ekström *et al.* 1989, Melin *et al.* 1991, Levy and Herzberg 1999). This reduced rate of hydrolysis may allow these FA to avoid immediate uptake by the adipose tissue and shuttle them into alternative metabolic pathways. Summers *et al.* (2000), however, found no *in vivo* selectivity of LPL action on chylomicron-TAG, in humans, which is in keeping with the *in vitro* findings of Morley and Kuksis (1977). Again, equivalent studies have

not been performed on marine carnivores but Levy and Herzberg (1999) showed this selectivity in rats accustomed to a fish oil diet. Despite this and considering the very high 20:5n-3 and 22:6n-3 content of the diets of marine carnivores (Ackman *et al.* 1980, Iverson *et al.* 1997b, Budge *et al.* 2002) it seems unlikely that a mechanism to selectively spare these FA from uptake by the adipose tissue would be necessary.

Fatty Acid Uptake and Esterification in Adipocytes

FA liberated from chylomicron TAG by adipose tissue LPL have two possible fates: uptake and esterification by adipocytes or release into venous plasma as non-esterified FA (Frayn *et al.* 1994, 1995). The rate of intracellular adipocyte TAG synthesis has recently been recognised as integral to the regulation of this branch-point (Frayn 1994, 1995). Because LPL achieves complete hydrolysis of chylomicron TAG, FA located at the *sn*-2 position are neither preferentially released to the plasma nor preferentially taken up by the adipose tissue (Summers *et al.* 1999). Summers *et al.* (1999) also conclude that, in humans, the metabolic events just after LPL hydrolysis of chylomicron TAG are largely unaffected by the nature of the FA within the TAG. The TAG used in their study, however, only contained significant amounts of 18:0 and 18:1n-9, making their comparison very narrow. Summers *et al.* (2000), in an *in vivo* study with a wider variety of dietary FA, also found no significant differences between FA in terms of their uptake into adipose tissue. It appears, therefore, that the metabolic events at this stage of FA incorporation into adipocytes should not influence the correlation of adipose tissue FA composition to that of the diet in any significant way.

Adipose Tissue Fatty Acid Flux

Hormone-sensitive lipase (HSL) is the other major enzyme responsible for adipose tissue FA flux. HSL is an intracellular neutral lipase highly expressed in adipose tissue. It is the rate-limiting enzyme in the release of non-esterified FA from adipose tissue and, as such, is thought to play a crucial role in providing the major source of energy for most tissues (Fredrikson *et al.* 1981, Holm and Østerlund 1999). HSL is capable of fully hydrolysing TAG to glycerol and non-esterified FA, but like most mammalian acylglycerol lipases, it shows a marked preference for the *sn*-1,3 ester bonds (Fredrikson *et al.* 1981, Fredrikson and Belfrage 1983). There is evidence that a distinct MAG lipase, which has little positional specificity, is the enzyme responsible for the hydrolysis of the *sn*-2 ester bond and that both this enzyme and HSL are necessary for complete hydrolysis *in vivo* (Tornqvist and Belfrage 1976, Fredrikson *et al.* 1986).

The net flow of FA into and out of the adipose tissue appears to be governed by concentration gradients created by the relative actions of HSL, LPL and the re-esterification pathway. It has long been recognised that HSL and LPL are regulated in a broadly reciprocal manner (Patten 1970) and Frayn *et al.* (1994, 1995) show that their regulation as well as that of the esterification pathway is highly co-ordinated with nutritional state. This co-ordinated regulation allows for the precise long-term matching of energy imbalance to adipose tissue TAG storage and mobilization.

Frayn *et al.* (1994, 1995) measured net exchange of non-esterified FA, TAG, and glycerol across a subcutaneous adipose depot in humans in both the post-absorptive and post-prandial states, producing the following observations. In the post-absorptive state, HSL is more active than LPL resulting in a net outward flux of FA across the capillary

wall. Total re-esterification (i.e. that due to re-esterification of both HSL- and LPL-derived FA) is low, causing 80-90% of the FA liberated from very low density lipoproteins (VLDL) and low density lipoproteins (LDL) by LPL to be released directly into the plasma. In the post-prandial state, the ratio of HSL to LPL action declines causing a net inward flux of FA across the capillary wall. Despite an increase in total re-esterification, 50-80% of LPL-derived FA are still released directly into the plasma. Results from several studies support the finding that a major proportion of LPL-derived FA escapes immediate retention (Eaton *et al.* 1969, Bergman *et al.* 1971, Scow 1977, Coppack *et al.* 1990, 1992, Karpe *et al.* 1992, Griffiths *et al.* 1994, Fielding *et al.* 1999).

Although the escape of a portion of the FA released by LPL may seem futile, two suggestions for its functional significance have been made. First, during the post-absorptive state, adipose tissue LPL may hydrolyse VLDL-TAG and release non-esterified FA into the circulation in order to provide energy for tissues whose LPL is less able to act on VLDL-TAG (Frayn 1995), such as human forearm muscle (Potts *et al.* 1991a, 1991b). Second, the escape of LPL-derived FA may be necessary in order to give precise control to the pathway of net fat storage by creation of a branch point (Frayn *et al.* 1994).

Some of the FA released by LPL into the venous plasma will be taken up by the liver and re-secreted as VLDL-TAG, giving rise to an inter-tissue TAG/FA cycle. The energy cost of such a cycle is relatively small (Elia *et al.* 1987, Wolfe *et al.* 1990), particularly since it does not involve esterification of FA in peripheral tissues, but this inter-tissue TAG/FA cycle means that FA can cycle through the body several times before ultimate utilization or storage. This escape of a large portion of dietary FA from

immediate retention and their subsequent cycling through the body could have a significant effect on the FA signature of adipose tissue in predators such as pinnipeds.

Despite the fact that Summers *et al.* (2000) found no evidence of differential metabolism of chylomicron TAG in terms of initial removal or uptake, the ultimate fate of the released FA may differ for individual FA. For example, the liver is the main site of peroxisomal chain-shortening of 22:1 FA (Norseth and Christophersen 1978), so those 22:1 FA that escape immediate retention and cycle back to the liver have the potential to be chain-shortened to their C_{20} and C_{18} counterparts. Considering the high levels of 22:1 FA in marine-based diets (Ackman and Hooper 1974), this recycling of dietary FA through the liver has the potential to affect the ultimate blubber FA signature of marine predators. Also, it appears that, in humans, the *in vivo* clearance rate of plasma 18:0 is lower than that of other FA (Hagenfeldt *et al.* 1972, Halliwell *et al.* 1996) and Hagenfeldt *et al.* (1972) suggest this is due to a reduced uptake of 18:0 by the liver. This could potentially make 18:0 more available for uptake by extra-hepatic tissues. This, however, does not appear to apply to adipose tissue of pinnipeds as the level of 18:0 found in the adipose tissue is at or below levels predicted from the diet (Kirsch *et al.* 2000, Cooper *et al.* 2001). Finally, the amount of 20:4n-6 and 20:5n-3 available for return to the adipose tissue could potentially be reduced by their role as major eicosanoid precursors. This is again unlikely to affect the blubber FA signatures of marine carnivores as 20:5n-3 and 22:6n-3 are extremely high in marine-based diets (Ackman *et al.* 1980). The small amount of the n-3 PUFA that would be lost to eicosanoid production could not have a significant effect of the overall levels of these FA.

Inter-prandial Remodelling of Adipose Tissue Triacylglycerol

Several researchers have suggested that interprandial remodeling, involving differential mobilization and re-esterification of FA, may also be important in determining the FA composition of adipose tissue. Early investigations into this question, which only considered a limited number and variety of FA, produced conflicting results. FA were found to be either randomly (Stein and Stein 1962, Spitzer *et al.* 1966, Nakamura *et al.* 1966) or selectively (Hollenberg and Douglas 1962, Hollenberg and Angel 1963, Hunter *et al.* 1970, Meinertz 1963) mobilized. More recent studies support the concept of selective mobilization with evidence of preferential loss of 18:3n-3 from human adipose during diet-induced weight loss (Phinney *et al.* 1990, Tang *et al.* 1993) and preferential release, *in vitro*, of several PUFA by rat HSL (Gavino and Gavino 1992). The work of Raclot and Groscolas (1993, 1995) established, through *in vitro* and *in vivo* experiments, that the mobilization of FA from adipose tissue is positively correlated with the level of unsaturation and negatively correlated with chain length. The presence of a double bond close the methyl end also increases the mobility of the FA. Raclot *et al.* (1995a,b) showed that this selective mobilization is not based on the positional distribution of FA within the TAG and that it appears to be a general characteristic of adipose tissue. Raclot and Groscolas (1995) showed that after a 56% depletion of total FA, 20% (22:1n-11) to 90% (20:5n-3) of the initial mass of individual FA was lost, corresponding to *in vivo* relative mobilization rates of 0.31 to 2.54. The same pattern and degree of selective mobilization was also found in *in vivo* studies of fasting Emperor penguins (Groscolas 1990). After an 80% depletion of initial FA, 20% (22:1n-11) to 94% (20:5n-3) of the individual FA was lost. Humans (Halliwell *et al.*

1996) and rabbits (Connor *et al.* 1996) have also been shown to exhibit similar patterns of selective mobilization.

The question of selective mobilization of individual FA from adipose tissue is extremely relevant to the use of blubber FA signatures to predict diet. Pinnipeds spend the majority of the year at sea and are only required to come ashore during the molting and breeding seasons. These periods of their life cycle are often associated with complete or partial fasts. Therefore, if selective mobilization of FA occurs to a large extent, the blubber FA signature would reflect the diet responsible for pre-fast fattening less and less as the fast proceeds. Current evidence available from pinnipeds is conflicting, as some studies have shown no preferential change in blubber FA stores during fasting, while others have suggested that some may occur in fasting lactating females (S.J. Iverson and L. Rea, unpublished data).

Summary and Thesis Objectives

There are a number of metabolic processes that can potentially affect adipose tissue FA composition and elucidating these is important to understanding the relationship between dietary and adipose tissue FA compositions in marine carnivores such as pinnipeds. The FA composition of adipose tissue, in humans, appears to predominantly reflect the early metabolic handling of individual FA (Summers *et al.* 2000). Attention should, therefore, be focused on determining whether or not this is also the case in marine carnivores. *De novo* FA synthesis is a function of dietary fat content and likely plays a more important role in determining adipose tissue FA composition in

predators such as pinnipeds than in those consuming higher-fat diets such as polar bears or humans eating a typical Western diet. The escape of a large portion of LPL-hydrolyzed dietary FA from immediate retention in the adipose tissue may also have a significant effect on the FA signature of adipose tissue in predators such as pinnipeds as the subsequent cycling of these FA through the body provides an opportunity for their modification or utilization. Finally, selective mobilization of individual FA has been demonstrated, *in vivo*, in several species (Groscolas 1990, Raclot and Groscolas 1995, Haliwell *et al.* 1996) whereas current evidence available from pinnipeds is conflicting (S.J. Iverson and L. Rea, unpublished data).

In my thesis research, I explore a number of aspects of FA metabolism in juvenile pinnipeds using several techniques including *in vitro* and *in vivo* radioisotope tracer methods, and captive feeding experiments. My specific objectives were to investigate 1) the *de novo* synthesis of FA in liver tissue, 2) the deposition and modification of individual dietary FA, 3) the relationship between dietary and chylomicron FA signatures, and 4) the relationship between dietary and blubber FA signatures when a completely homogenous diet of known FA signature is consumed. Through controlled feeding experiments I was also able to study characteristics of nutrient and energy balance in juvenile pinnipeds.

In order to assess the products of *de novo* FA synthesis in pinnipeds I incubated grey seal liver tissue in media containing isotopically labelled precursors. Radioisotope tracers are also commonly used to study the *in vivo* metabolism of individual FA (Owen *et al.* 1975, Thomassen *et al.* 1985, Hjelte *et al.* 1990, Linares and Henderson 1991, Green and Yavin 1993, Rabinowitz and Myerson 1994, Nilsson *et al.* 1996). I employed

this method to study the deposition, and potential modification, of a specific dietary FA in the blubber of juvenile grey seals as well as the liver and adipose tissue of adult mink (*Mustela vison*). Mink were used as model animals because they, too, are carnivores accustomed to fish based diets (Linscombe *et al.* 1982, Tolonen 1982), but their much smaller size avoids the problem of extreme signal dilution.

As described above, differential metabolism of individual FA throughout digestion, absorption and incorporation into chylomicrons can influence the relationship between the FA composition of the diet and that in the adipose tissue. To investigate the metabolism of individual dietary FA at these early stages of assimilation, I characterized the FA composition of chylomicrons over the complete digestion period in grey seals and compared these with that of the meal consumed. I also investigated the capacity of chylomicron FA signatures to provide quantitative estimates of diet composition using QFASA.

Controlled feeding studies have proven useful in the investigation of FA metabolism and the development of calibration coefficients (Iverson *et al.* 2004). In previous controlled feeding experiments, seals were fed individual fish and FA were analyzed from a sub-sample of these fish (Kirsch *et al.* 2000, Tollit *et al.* 2003, Iverson *et al.* 2004). However, individual fish of a species can vary substantially in FA composition and fat content (Jangaard 1974, Montevecchi and Piatt 1984, Mårtensson *et al.* 1996, Budge *et al.* 2002, Iverson *et al.* 2002) such that the exact dietary intake of FA has not been known. I performed a series of controlled feeding experiments in which completely homogenous fish-based diets were intubated to juvenile grey seals and the FA composition of the diets determined. This allowed precise knowledge of the FA

composition of the diet being consumed by the seals, and a solid understanding of the relationship between dietary and blubber FA signatures. Changes in mass and body composition measured throughout these captive feeding experiments provided insight into nutrient and energy balance in juvenile grey seals.

Chapter 2. Studying Lipid Metabolism using Radioisotope Tracers: *in vivo* and *in vitro* studies

Introduction

Within the entire suite of FA present in pinniped blubber, individual FA vary in the degree to which they reflect diet. Certain FA may be designated as solely exogenous and others as endogenous in origin, whereas others are a result of contributions from both sources. Short chain FA ($\leq$ 12 carbons) released from TAG in the stomach and small intestine can partition into the aqueous phase and are transported, bound to albumin, in the portal vein to the liver where they are oxidized (Jackson 1974, Brindley 1985). Thus, any short chain FA found in the blubber are of endogenous origin and not useful in an evaluation of diet. Other FA, such as those of the n-3 and n-6 families, can only be of exogenous origin (Cook 1991) and clearly contain important information regarding the types of prey consumed. FA that can arise from both endogenous and exogenous sources include 16:0 and 18:0 and their immediate unsaturates, 16:1n-7 and 18:1n-9. These FA are readily synthesized by the animal as well as consumed in the diet. Other FA in this category are those that are modification products of FA originally of dietary origin (e.g. 22:5n-3). A detailed understanding of patterns of FA metabolism and biosynthesis is, therefore, important to the use of blubber FA signatures in estimating pinniped diets.

In Vitro Assessment of Fatty Acid Synthesis

The complete sequence of steps in FA synthesis was elucidated in the 1960s and 1970s (Wakil *et al.* 1958, Lynen 1967, Vagelos and Larrabee 1967, Tanabe *et al.* 1975,

Arslanian *et al.* 1976, Buchner and Kolattukudy 1976, Stoops *et al.* 1976, Smith and Stern 1979). The immediate substrate used in *de novo* FA synthesis is acetyl-CoA. Free 16:0 is the primary product released from the FA synthase complex (Volpe and Vagelos 1973, Brindley 1978). Some 14:0 and 12:0 may also be formed, as well as a trace of 18:0 (Wakil *et al.* 1983). This *de novo* FA synthesis occurs in the cytosol so as to separate it from the degradative process of FA oxidation occurring in the mitochondria.

Synthesized FA may be deposited or utilized as is, or they may go on to be dehydrogenated to form unsaturated FA and/or elongated to form longer chain FA. FA chain elongation occurs by the addition of two-carbon units in a reaction similar to *de novo* synthesis. Enzymes catalyzing FA elongation and desaturation are located on the endoplasmic reticulum (Allen 1976, Cook 1991). The Δ9 desaturase (the Δ symbol is used to refer to the number of carbon atoms from the terminal carboxyl end) is usually the predominant, if not exclusive, desaturation enzyme for SFA. The formation of PUFA follows by further oxidative desaturation. Animals cannot insert double bonds beyond the Δ9 position. The other desaturase enzymes possessed by animals introduce double bonds at the Δ6 and Δ5 positions.

The liver is the primary site for lipid metabolism (Volpe and Vagelos 1973, Bloch and Vance 1977, McGarry and Foster 1980) and is the organ most susceptible to dietary induced changes in metabolism. Depending on the species, adipose tissue can also be a major organ system in which FA synthesis occurs when excess calories are consumed (Leveille *et al.* 1975, Hollands and Cawthorne 1981). Virtually all investigations into *de novo* FA synthesis and modification have been carried out on laboratory animals (mostly rodents) and humans. Thus one aim of this study was to directly determine the products

of *de novo* FA synthesis and modification in pinnipeds by incubating liver and blubber tissue in media containing isotopically labelled precursors.

In Vivo Fatty Acid Metabolism

Many studies have used radio-labelled FA to show that individual FA can experience differential absorption, incorporation into chylomicron lipids and eventual deposition in adipose tissue (Ockner *et al.* 1972, Bergstedt *et al.* 1990, Hjelte *et al.* 1990, Bernard and Carlier 1991, Nilsson *et al.* 1992, Nilsson *et al.* 1996). Other studies have shown that oxidation of dietary FA can be influenced by the chain length and degree of saturation of the individual FA (Jones *et al.* 1985, Leyton *et al.* 1987). Another use of radio-labelled FA has been the investigation of the modification products of individual FA in different species and within different body compartments (Owen *et al.* 1975, Thomassen *et al.* 1985, Linares and Henderson 1991, Green and Yavin 1993, Rabinowitz and Myerson 1994). Modification of dietary FA has been proposed to occur in pinnipeds, however, this suggestion is based on circumstantial evidence (e.g., Ackman *et al.* 1971, Ackman & Hooper 1974) and has never been directly studied.

Pinnipeds present a significant problem in tracking ingested labelled FA. They are large in body size and blubber constitutes a high percent of body mass (approximately 10-45%; Worthy and Lavigne 1987, Ryg *et al.*1990, Iverson *et al.* 1995, Arnould *et al.* 1996, Aarseth *et al.* 1999, Kirsch *et al.* 2000), both of which result in a large dilution of the labelled FA. The cost of feeding sufficient amounts of such labelled compounds can be quite high. One way to circumvent this logistical problem is to use a much smaller, model animal. A second approach, developed in the course of this thesis (Budge *et al.*

2004), is to employ a more sensitive method of analysis that is capable of identifying labelled FA in the blubber of pinnipeds when small doses (< 1mCi) of labelled lipid are ingested. In this previous study (Budge et al. 2004), we gained insight into the fate of ingested radio-labelled 16:0 and 18:1 in juvenile grey seals.

In the present study, I employed both labelling approaches using mink (*Mustela vison*) as a much smaller model carnivore, also accustomed to fish based diets (Linscombe *et al.* 1982, Tolonen 1982), and juvenile grey seals. For this study, I investigated the fate of ingested ^{3}H-labelled cetoleic acid (22:1n-11). 22:1n-11 has proven to be an important FA when using adipose tissue FA composition to study diet in marine mammals and seabirds (Iverson 1993, Kakela *et al.* 1993, Smith *et al.* 1996, Iverson *et al.* 1997a,b, Raclot *et al.* 1998, Brown *et al.* 1999, Dahl et al. 2000). Although vertebrates can theoretically synthesize 22:1n-11, this FA primarily originates from the fatty alcohols (wax esters) of certain copepod species (Lee *et al.* 1971, Pascal and Ackman 1976, Ackman *et al.* 1980, Falk-Petersen *et al.* 1990). The concentration of this FA also varies widely among different fish and invertebrate species (Ackman 1980, Iverson 1993, Dahl *et al.* 2000, Budge *et al.* 2002, Iverson *et al.* 2002), making 22:1n-11 a good indicator of diet when found in the predator. However, feeding studies have shown that the 22:1 isomers are under-represented in adipose tissue relative to the diet (Holland *et al.* 1990, Lin and Connor 1990, Lin *et al.* 1993, Kirsch *et al.* 1998, Kirsch *et al.* 2000, Cooper *et al.* 2001, Iverson *et al.* 2004). This implies that metabolism within the predator has a particularly strong influence on the relationship between the concentration of this FA in the diet and in the adipose tissue. I investigated the

modification and deposition of labelled 22:1n-11 *in vivo* to provide insight into this relationship and to better understand the origin and fates of adipose tissue FA.

Administering a differently labelled control FA in addition to ^{3}H-labelled 22:1n-11 allows for comparison between the levels of deposition of the two FA, giving a quantitative measure of relative recovery. ^{14}C-labelled 18:1n-9 was chosen as a control FA because it was expected to experience little modification between ingestion and final deposition in the adipose tissue (Cook 1991, Budge *et al.* 2004) and because its metabolism is generally representative of the dietary FA pool as a whole (Hagenfeldt *et al.* 1972, Jones *et al.* 1985, Wang and Koo 1993, Jones 1994). Thus, this measure of relative recovery should indicate the degree to which the under-representation of 22:1n-11 in the adipose tissue relative to the diet is a direct consequence of its metabolism.

Methods

In Vitro Experiments

Animal Maintenance and Sampling.

Ten juvenile (~ 6 mos.) grey seals were caught on the beaches of Sable Island, Nova Scotia, Canada. They were placed in large, covered pens where an initial health assessment was made by Dr. Chris Harvey-Clark, Dalhousie University Veterinarian. Blubber biopsies were performed according to Kirsch *et al.* (2000). Liver biopsies were then performed. Animals were first injected with 5-10 mg valium in the epidural vein. A local anaesthetic (3 cc 2% lidocaine) was administered to the right paracostal area in an

inverted L block. The pelage was then scrubbed with a mixture of betadine and sterile K-Y jelly, to sterilize and part the hair. A 2 mm stab incision was made posterior to the border of the 8th rib on the lateral surface of the animal with an #11 scalpel blade to facilitate passing the biopsy instrument (16G x 20 cm Coaxial Quick-Core™ biopsy needle, Cook (Canada) Inc.) through the skin. This instrument consists of an inner bevelled point stylet, with a 20 mm specimen notch, and a spring loaded outer cannula. The biopsy needle was directed in a craniomediodorsal direction into the upper aspect of the right lateral lobe of the liver. Once the inner stylet of the needle advanced into the liver parenchyma, the outer cannula was advance over the inner rod. Duplicate 40 mg liver samples were collected in this fashion. A broad spectrum long acting antibiotic (Procain penicillin G 40,000 units/kg) was administered for antibiotic prophylaxis. The animals were observed for 24 hours after biopsy and then released.

Fatty Acid Synthase Assay

Upon sampling, blubber and liver tissue samples were immediately transferred to liquid nitrogen (-195°C), to essentially stop all enzyme activity. It was deemed to be safest to keep liver and blubber samples for *in vitro* enzyme analyses in liquid nitrogen as we did not have access to a -80°C freezer. The first analyses were conducted on liver samples. Due to events beyond my control, the liquid nitrogen store failed prior to analysis of the blubber samples and all were ruined.

The FA synthetase assay protocol was used for liver samples only and was based on the procedures of Ysu *et al.* (1969). The reaction mixture consisted of 35 µl potassium phosphate buffer (pH 6.8, 2.8 M), 50 µl 2-Mercaptoethanol (0.1 M), 30 µl

EDTA (0.1 M), 50 µl malonyl-CoA (1mM), 5 µl ^{14}C acetyl-CoA (0.5 mM, Dupont NEN), 20 µl acetyl-CoA (0.5 mM) and 30 µl NADPH (10mM, prepared daily). The incubation mixture was equilibrated for 5 minutes at 37°C in a shaking water bath prior to the addition of the crude enzyme solution. Approximately 0.01 g of liver tissue was homogenized in a 1 ml plastic microcentrifuge tube with 0.8 ml of 0.25 M potassium phosphate buffer containing 1 mM dithiothreitol. The enzyme preparation was added to start the reaction, giving a final assay volume of 1 ml. After incubation for 15 minutes at 37°C, the reaction was stopped by the addition of 1 ml HCl. This drops the reaction pH and denatures enzyme proteins.

Lipid extraction proceeded by the addition of 1 ml of absolute ethanol and 2 ml petroleum ether. After phase separation the upper (ether) phase was transferred to a new pre-weighed test tube. The lower aqueous phase was then extracted two more times with petroleum ether (2 ml). The petroleum ether extracts were evaporated to dryness under nitrogen and weighed.

In Vivo Experiments

Isotopically Labelled Fatty Acids

22:1n-11 is not commercially available so it was necessary to isolate it from surplus FA methyl ester (FAME) samples prior to it being sent to Perkin Elmer for tritium labelling. Isolation was achieved through argentation thin layer chromatography (TLC) and reverse phase high performance liquid chromatography (HPLC) according to the methods detailed in Budge et al. (2004). A sample of 50 mg of isolated 22:1n-11 was

required by Perkin Elmer to ensure an appropriate yield of labelled product. The HPLC sample throughput was constrained by the column bore, which allowed the injection of only 1mg FAME peak^{-1}. Thus, isolation of such a large volume of 22:1n-11 required many injections. [1-^{14}C]-oleic acid was purchased from Dupont NEN.

Animal Maintenance and Sampling: Mink Experiment

Five adult male mink, from the Nova Scotia Agricultural College (NSAC) Fur Unit, were fed 1 mCi ^{3}H-labelled 22:1n-11 and 0.01 mCi ^{14}C-labelled 18:1n-9 using an eyedropper in combination with a 100 g meal of fish. After a 6 h or 9 h incubation period, the mink were anaesthetized by intra-muscular injection of ketamine hydrochloride at 25mg kg^{-1} body weight. The animals were then euthanised by intra-cardiac injection of sodium pentobarbital at 0.44 ml kg^{-1} body weight. Tissue samples, weighing approximately 1 g, were then taken from the liver as well as the mesenteric, omental, perirenal, inguinal, and subcutaneous adipose depots. For simplicity of presentation, the data for the inguinal, omental and perirenal adipose depots were averaged to form the visceral category. Samples were placed in glass vials (with Teflon lined caps) with chloroform and 0.01% BHT, then frozen until further lipid analysis was possible.

Animal Maintenance and Sampling: Seal Experiments

Two free-ranging juvenile grey seals were captured on Sable Island, Nova Scotia, placed in a fenced enclosure on the beach and fasted for approximately 12 h. Each animal was then fed 1.5 mCi ^{3}H-labelled 22:1n-11 and 0.1 mCi ^{14}C-labelled 18:1n-9 by

gastric intubation. Budge *et al.* (2004) found that administering 0.5 mCi of labelled FA was sufficient to produce a detectable signal in the blubber. However, because 22:1n-11 typically experiences reduced deposition relative to other FA, I chose to use three times as much radioactivity in this experiment. On the other hand, 18:1n-9 is expected to experience a relatively direct deposition, so a quantity of only 0.1 mCi of ^{14}C-labelled 18:1n-9 was used. A 24 h incubation period was chosen in light of the very low level of absolute deposition (< 2%) of ingested ^{3}H-labelled triolein found by Budge *et al.* (2004) using a 12 h incubation period. Blubber biopsies were taken from both the right and left flank of each animal according to Kirsch et al. (2000) totalling approximately 0.5 g per animal. Samples were placed in glass vials (with Teflon lined caps) with chloroform and 0.01% BHT and frozen until further lipid analysis was possible. The animals were then released.

Lipid Analysis

Lipids were extracted from adipose samples according to a modified Folch *et al.* (1957) procedure described in detail in Iverson *et al.* (2001). Briefly, samples were extracted with 2:1 chloroform:methanol and dried over anhydrous sodium sulphate. The extracted lipid, from all *in vitro* and *in vivo* experiments were converted to FAME, according to Morrison and Smith (1964), by reaction of approximately 100 mg of lipid with 1.5 mL of boron trifluoride in methanol (8% v/v) and 1.5 mL of hexane. The mixture was heated at 100°C for 1 hour in a nitrogen atmosphere and FAME were extracted with hexane. Individual FA were isolated from FAME samples according to

Budge *et al.* (2004). Briefly, FAME samples were separated by degree of unsaturation using argentation TLC. The FAME of each fraction were subjected to reverse phase HPLC and individual FAME were manually collected in glass test tubes. The purity of each isolate was assessed using temperature-programmed gas liquid chromatography according to Iverson *et al.* (1997b), on a Perkin Elmer Autosystem II Capillary FID GC equipped with a flexible fused silica column (30 m x 0.25 mm ID) coated with 50 % cyanopropyl polysiloxane (0.25 μm film thickness; J & W DB-23; Folsom, CA, USA) and linked to a computerized integration system (Turbochrom 4.1 software, PE Nelson). FAME were identified by comparison of retention times with known standards (Nu Check Prep, Elysian, MN, USA). Each FAME was then mixed with scintillation cocktail (ScintiVerse II) and counted in a Beckman Scintillation Counter (LS3801).

Results

In Vitro Experiments

As stated above, only liver samples were able to be assayed for FA synthetase activity. PUFA fractions were not further separated into individual FA because as a whole this fraction showed no sign of radioactivity. The primary product of the FA synthase enzyme complex from grey seal liver was clearly 16:0, although some label also appeared in 18:0 (Figure 2.1).

In Vivo Experiments

^{3}H-labelled 22:1n-11

The relative recovery of 22:1n-11 was calculated as the ratio of ^{3}H 22:1n-11 to ^{14}C 18:1n-9 upon recovery in a depot, divided by the ratio of ^{3}H 22:1n-11 to ^{14}C 18:1n-9 ingested. In the mink that had a 6 h incubation period, there was a large degree of individual variation in the relative recovery of 22:1n-11 (Figure 2.2). In general, Mink 1 had the greatest relative recovery, with its highest value being 0.60 in the mesenteric adipose tissue, while Mink 3 had the lowest relative recoveries, < 0.11 in all tissues. In all 6 h mink, however, the mesenteric adipose depot showed a greater relative recovery of 22:1n-11 compared to the other adipose depots and the liver. In the mink with a 9 h incubation period, the relative recovery was similar for both animals and was similar across all tissues sampled. In all tissues, the relative recovery of 22:1n-11 was less at 9 h than it was after a 6 h incubation period. In Seal 1 and 2 the relative recovery of 22:1n-11 in blubber was, at 0.67 and 0.84, respectively, higher than in any of the mink tissues.

The concentrations of ^{3}H radioactivity present in the specific FA isolated from the different mink tissues indicate considerable individual variation in the incorporation of the ^{3}H label (Table 2.1). Generally speaking, the concentration of ^{3}H radioactivity in the mink incubated for 6 h was greater in individual FA than that in mink incubated for 9 h. At both 6 h and 9 h the concentration of ^{3}H 22:1 in the liver was greater than in any of the adipose depots (linear mixed effects model, $p < 0.044$). Among the adipose depots, the mesenteric adipose tissue had a significantly greater concentration of the originally fed ^{3}H 22:1 than either the subcutaneous or visceral depots at 6 h (linear mixed effects model, $p < 0.003$) but not 9 h. Levels of all other radioactive FA recovered were quite variable.

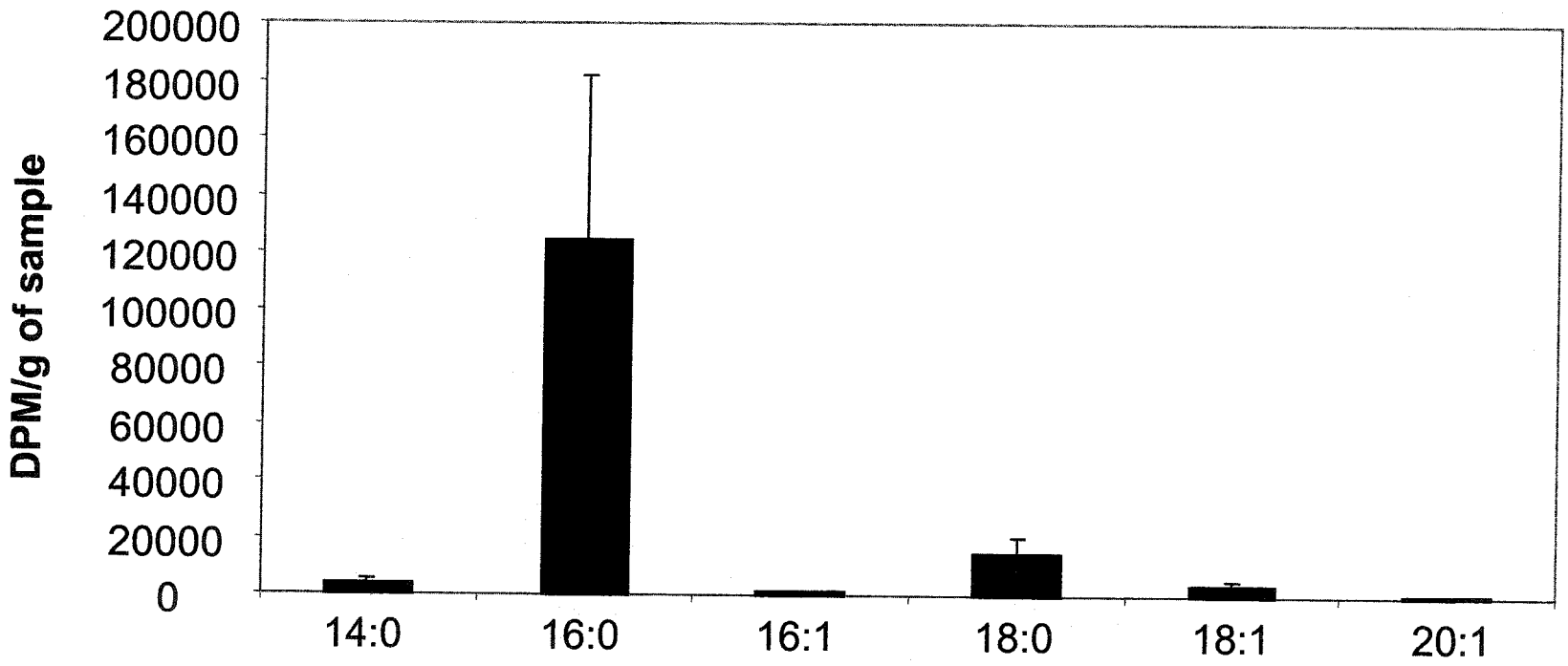

Figure 2.1: Radioactivity measured in *de novo* synthesized fatty acids isolated from *in vitro* incubation of grey seal (*Halichoerus grypus*) liver tissue with ^{3}H-labelled acetyl-CoA. Values are averages ± 1 SE (n = 10)

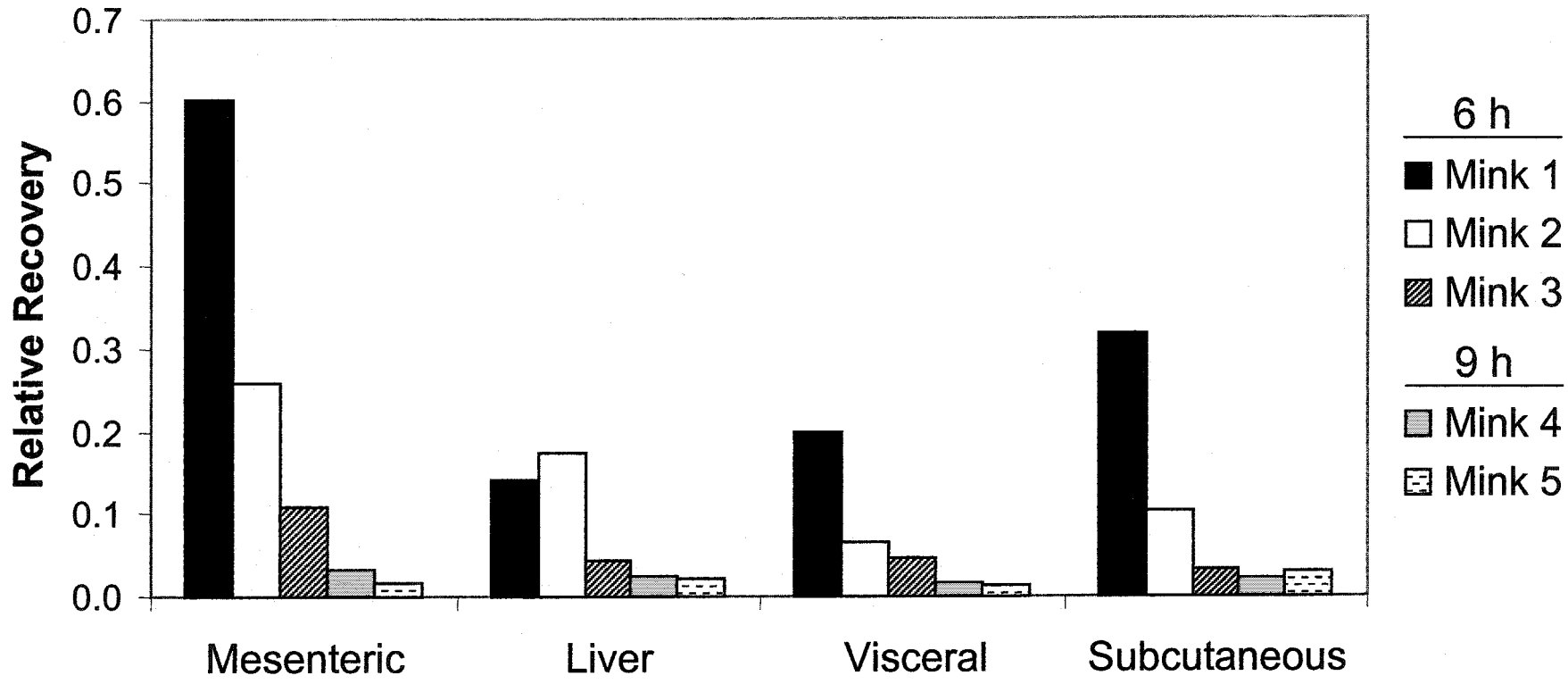

Figure 2.2: Relative recovery [(^{3}H22:1$_{recovered}$/^{14}C18:1$_{recovery}$)/(^{3}H22:1$_{ingested}$/^{14}C18:1$_{ingested}$)] of labelled fatty acids in adipose tissue depots and liver of adult male mink fed 1 mCi ^{3}H-labelled 22:1n-11 and 0.01 mCi ^{14}C-labelled 18:1n-9 after a 6 h or 9 h incubation period.

Table 2.1: ^{3}H radioactivity recovered in fatty acids isolated from various mink tissue sampled at either 6 h (n = 3) or 9 h (n = 2) post-feeding. Values are 1000 x dpm/g lipid ± CV.

	Mesenteric		Liver		Visceral		Subcutaneous	
Fatty Acid	6 h	9 h	6 h	9 h	6 h	9 h	6 h	9 h
14:0	5.3 ± 1.30	0.2 ± 0.77	233.9 ± 0.80	25.0 ± 0.56	4.2 ± 0.62	0.1 ± 0.69	2.5 ± 1.12	0.1 ± 1.41
16:0	10.5 ± 0.85	1.2 ± 1.17	308.7 ± 0.72	352.1 ± 0.96	10.3 ± 0.26	0.6 ± 0.89	6.2 ± 0.41	0.4 ± 0.91
18:0	9.4 ± 0.91	1.0 ± 1.08	406.7 ± 0.75	360.5 ± 1.01	11.2 ± 0.08	0.6 ± 0.53	8.8 ± 0.66	0.3 ± 0.75
16:1	2.4 ± 0.33	0.6 ± 1.08	97.4 ± 1.15	19.5 ± 0.70	4.1 ± 0.50	0.5 ± 1.07	4.9 ± 1.37	0.4 ± 1.00
18:1	53.1 ± 1.52	3.4 ± 1.21	83.9 ± 0.75	68.6 ± 0.55	7.9 ± 0.44	1.9 ± 1.14	14.2 ± 1.33	1.2 ± 1.14
20:1	2.1 ± 0.69	0.4 ± 1.07	36.6 ± 0.77	22.6 ± 0.76	3.0 ± 0.35	0.2 ± 0.78	2.1 ± 1.03	0.2 ± 0.68
22:1	14.7 ± 0.61	0.7 ± 1.17	65.8 ± 0.70	21.9 ± 0.29	1.7 ± 0.17	0.2 ± 0.76	1.7 ± 0.40	0.2 ± 0.41
Total	97.4 ± 1.18	7.4 ± 1.15	1233.0 ± 0.74	870.2 ± 0.91	42.4 ± 0.17	4.0 ± 0.95	40.3 ± 0.94	2.8 ± 0.97

To account for the large amount of individual variation in the absolute concentrations of radioactivity and to make the data comparable across individuals, the radioactivity in each FA was expressed as a percent of the total ^{3}H measured in a specific tissue. Even after standardizing the radioactivity data, individual differences in the metabolic processing of the ingested ^{3}H 22:1n-11 were apparent in some depots after a 6 h incubation period. For example, the mesenteric adipose tissue of Mink 3 contained the majority of its radioactivity in 18:1 (63.7%) and relatively little remaining in 22:1 (10.3%), whereas that of Mink 2 had a relatively large amount of its radioactivity remaining in 22:1 (38.1%) and a smaller amount found in 18:1 (18.6%) (Figure 2.3).

The distribution of radioactivity among the FA in the visceral and subcutaneous adipose depots of the 6 h minks showed somewhat different patterns from that of the mesenteric adipose tissue of the same animals (Figure 2.3). In both the visceral and subcutaneous adipose depots little of the radioactivity in any of the mink was remaining in 22:1 (< 7%). Somewhat less radioactivity is found in the MUFA of the visceral (34.4%) and subcutaneous (39.5%) depots compared to the mesenteric depot (44.0%), whereas much more of was found in the SFA (average of 61.4%, 54.3% and 31.7%, respectively). The distribution pattern of radioactivity among the FA in the liver was quite different from that of the adipose depots in that the vast majority of the ^{3}H recovered was located in the SFA (average of 76.1%). At 9 h, the distribution patterns of radioactivity in all adipose depots were similar, with SFA accounting for an average of 35.2%, 39.6% and 30.7%, and MUFA accounting for an average of 56.1%, 54.5% and 58.4%, for the mesenteric, visceral and subcutaneous depots, respectively (Figure 2.4). The liver tissue sampled after a 9h incubation period maintained a ^{3}H distribution pattern

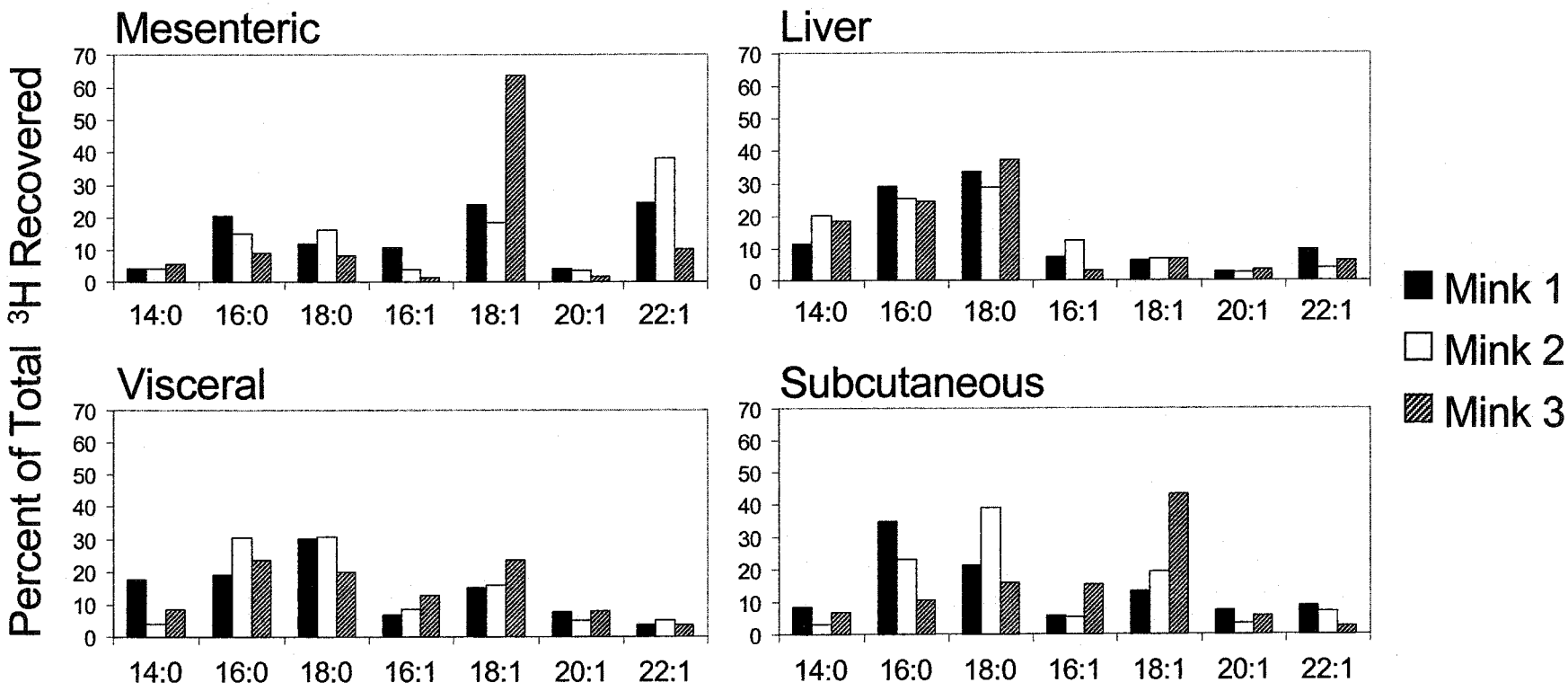

Figure 2.3: Percent of total ^{3}H radioactivity recovered found in each fatty acid isolated from adipose tissue depots and liver of adult male mink fed 1 mCi ^{3}H-labelled 22:1n-11 and sampled after a 6 h incubation period.

that was distinct from the adipose depots in its abundance of radioactivity in the SFA (average of 80.7%).

Comparison of these data from 6 h and 9 h incubated animals revealed several interesting findings (Figures 2.3 and 2.4). First, the proportion of the radioactivity in 22:1 in the mesenteric adipose was higher at 6 h compared to 9 h, where its level is equivalent to that found in the visceral and subcutaneous adipose depots of both treatment groups. Second, the distribution pattern of radioactivity in the FA of the liver was virtually identical in the 6 h and 9 h mink. Third, in both the visceral and subcutaneous adipose, the proportion of ^{3}H radioactivity in 18:1 was greater in the 9 h mink (average of 38.8% and 37.3%, respectively) than in the 6 h mink (average of 18.2% and 25.3%, respectively). This was coupled with a general reduction in the amount of ^{3}H radioactivity found in the SFA at 9 h relative to 6 h for both the visceral (average of 39.6% vs. 61.4%) and subcutaneous depots (average of 30.7% vs. 54.3%).

Similar to findings for mink, significant amounts of ^{3}H were found in each of the SFA and MUFA isolated from the seal blubber samples (Table 2.2). Tritium recovery, on a per gram blubber basis, was comparable in the two seals with Seal 1, the smaller of the two at 42.0 kg vs. 50.5 kg, showing a 1.4 fold greater concentration. However, since body composition measurements were not taken from these animals, the absolute dilution of the ingested radioactivity is not known. Figure 2.5 illustrates the percent distribution of the total recovered ^{3}H among the isolated FA. The FA with the largest proportion of total recovered radioactivity was 18:1 in both Seal 1 and Seal 2 (37.8% and 43.1%, respectively). Seal 2 had a larger proportion of its total radioactivity remaining in 22:1

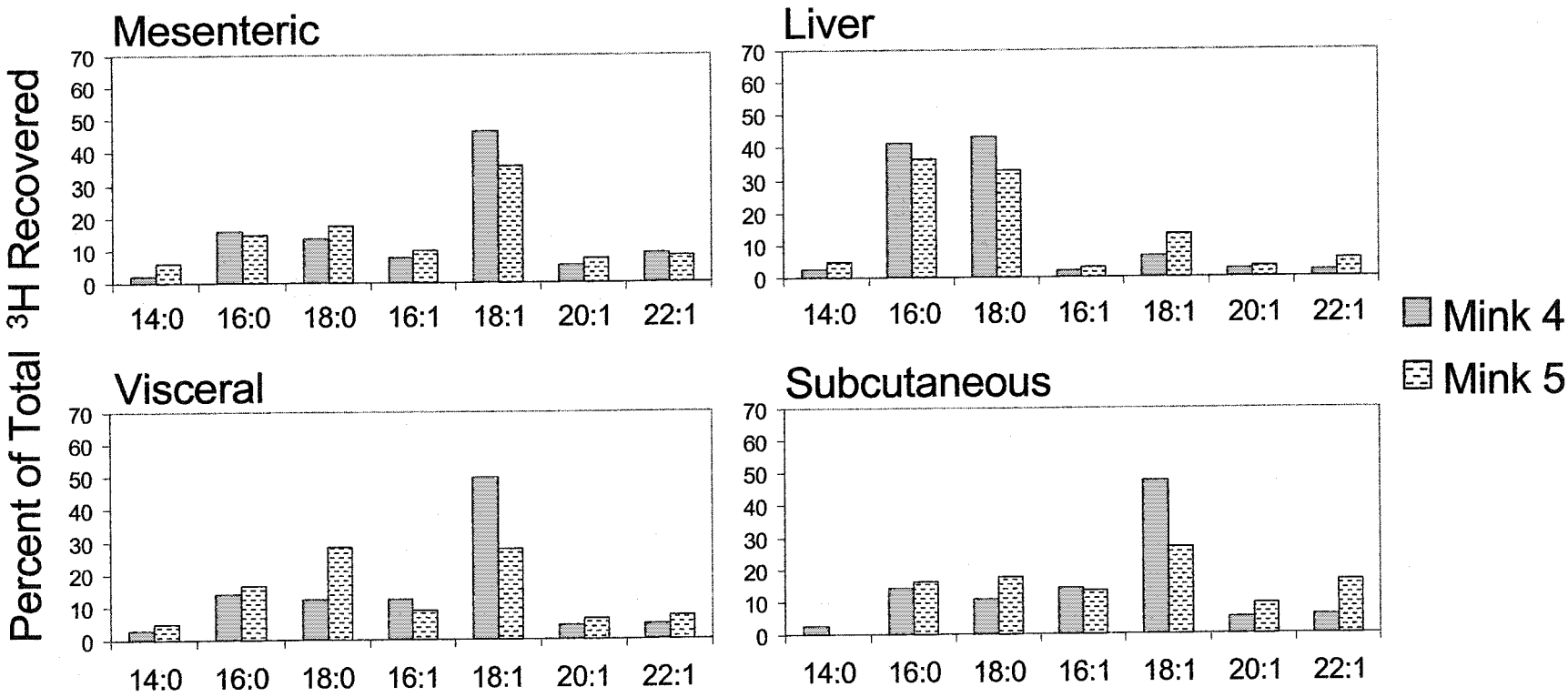

Figure 2.4: Percent of total ^{3}H radioactivity recovered found in each fatty acid isolated from adipose tissue depots and liver of adult male mink fed 1 mCi ^{3}H-labelled 22:1n-11 and sampled after a 9 h incubation period.

Table 2.2: ^{3}H radioactivity recovered in fatty acids isolated from juvenile grey seal blubber samples. Values are 1000 x dpm/g blubber.

	^{3}H dpm/g blubber	
Fatty Acid	Seal 1	Seal 2
14:0	7.7	3.8
16:0	3.2	2.0
18:0	11.0	1.2
16:1	6.4	2.2
18:1	30.0	31.0
20:1	6.1	2.8
22:1	8.8	9.0
Total	73.2	52.0

(23.0% vs. 12.6%) while Seal 1 had a greater proportion of its total radioactivity in the various saturates (32.6% vs. 19.3%).

To assess the extent to which mink were useful animal models for the investigation of the metabolism of marine lipids by a pinniped, I compared the distribution of ^{3}H radioactivity among the FA in the subcutaneous adipose depot of mink and the blubber of seals (Figure 2.6). The small sample size and variation within each of the treatment groups prevent firm conclusions, nevertheless, the proportion of radioactivity recovered from each FA in the seals was similar to the proportions found in the 6 h and 9 h mink.

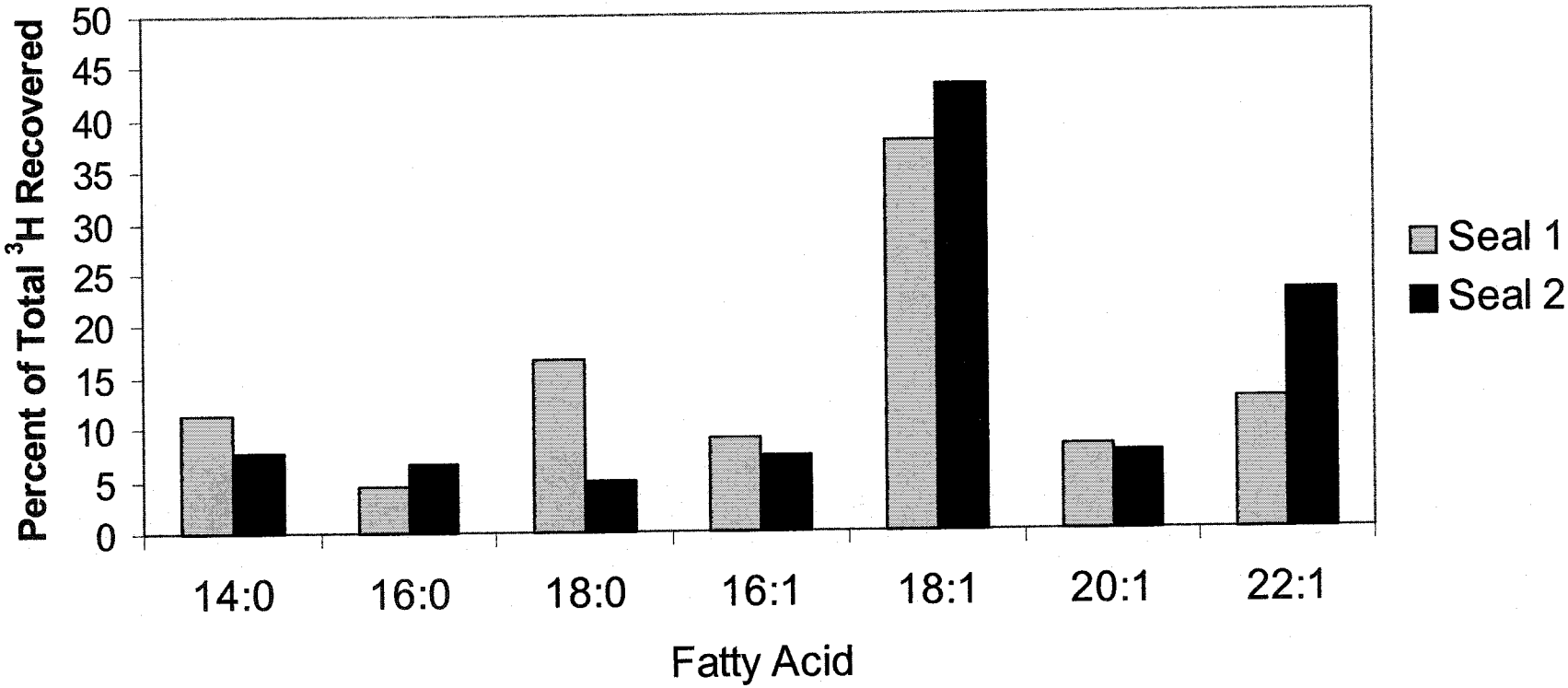

Figure 2.5: Percent of total ^{3}H radioactivity recovered found in each fatty acid isolated from the blubber of grey seals fed 1.5 mCi ^{3}H-labelled 22:1n-11 and sampled after a 24 h incubation period.

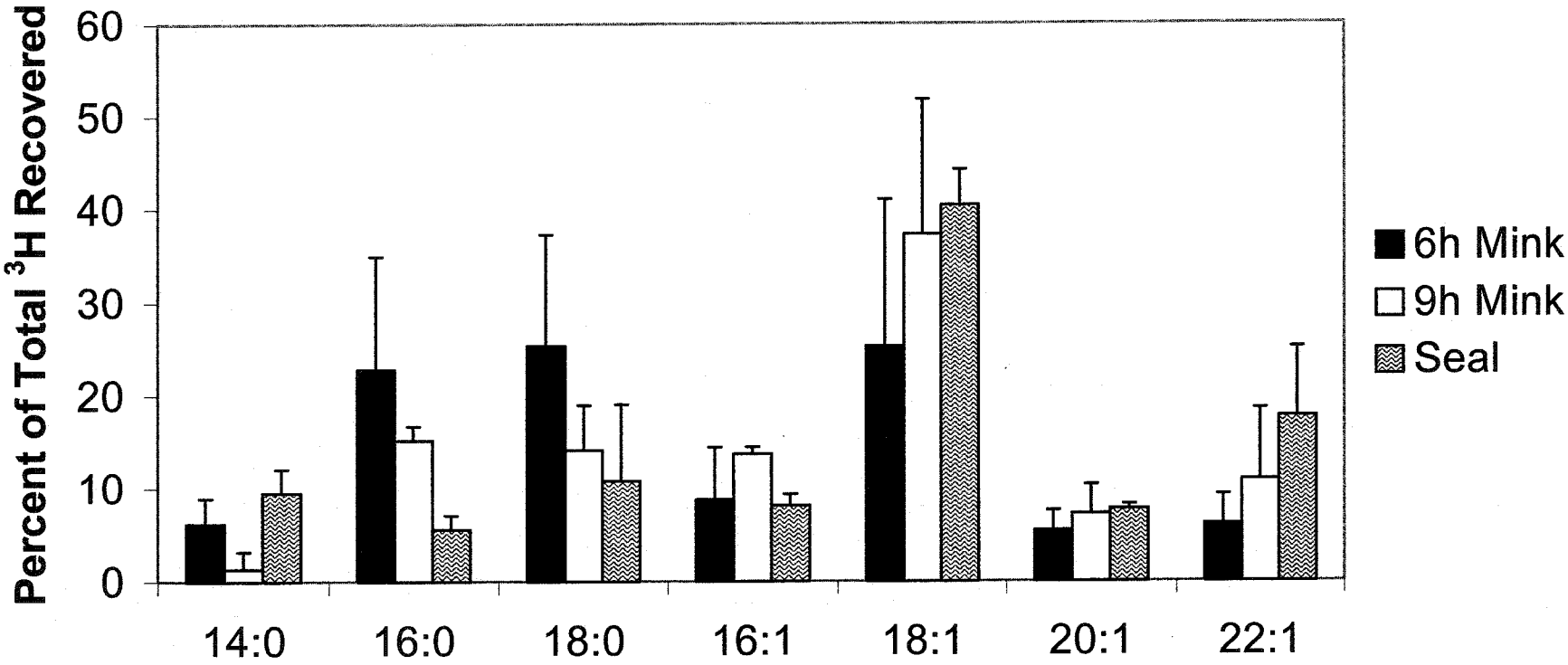

Figure 2.6: Percent of total ^{3}H radioactivity recovered found in each fatty acid isolated from the subcutaneous adipose tissue of adult male mink fed 1 mCi ^{3}H-labelled 22:1n-11 and sampled after a 6 h or 9 h incubation period and the blubber of grey seals fed 1.5 mCi ^{3}H-labelled 22:1n-11 and sampled after a 24 h incubation period. Values are averages ± 1 SD.

Discussion

In Vitro Experiments

Consistent with results from rats, 16:0 was the primary product of FA synthesis in grey seal liver tissue (Volpe and Vagelos 1973, Brindley 1978, Figure 2.1). Wakil *et al.* (1983) found that, in rats, some 12:0, 14:0 and traces of 18:0 may also be formed by the FA synthase enzyme complex. The FA produced in seal liver with the next highest level of radioactivity was 18:0. While much lower than the level found in 16:0 it was clearly detectable. At present it is not possible to assess whether the labelled 18:0 detected was a direct product of the FA synthase enzyme complex or a product of subsequent chain elongation.

In Vivo Experiments

The 22:1 FA are generally under-represented in adipose tissue relative to the diet (Holland *et al.* 1990, Lin and Connor 1990, Lin *et al.* 1993, Kirsch et al. 1997, Kirsch *et al.* 1998, Kirsch *et al.* 2000, Cooper *et al.* 2001, Iverson *et al.* 2004). It has been suggested that the lower levels of 22:1 in depot TAG are a result of poor digestibility and lower esterification rates of the 22:1 FA (Thomasson 1956, Caselli *et al.* 1979). The most important factor governing the observed levels of these FA in depot TAG, however, is presumably the peroxisomal chain shortening of 22:1 FA (Bremer and Norum 1982). Animals that are unaccustomed to consuming large amounts of 22:1 FA have a limited

capacity for their metabolism, ultimately resulting in an intracellular cardiac lipidosis which causes a deterioration of myocardial function (see Bremer and Norum 1982 for a review). This cardiac lipidosis is, however, temporary because peroxisomal β-oxidation is induced by the intake of 22:1 FA-containing diets (Christiansen *et al.* 1979b, Thomassen *et al.* 1979, Neat *et al.* 1980, Neat *et al.* 1981, Thomassen *et al.* 1985). As might be expected, animals accustomed to diets high in 22:1 FA are better able to chain-shorten these FA, thus avoiding any potentially harmful cardiac lipidosis and leading to an even greater under-representation of these FA in adipose depots relative to the diet. For example, Rouvinen and Kiiskinen (1989) showed that mink, whose wild diet is predominantly fish based (Linscombe *et al.* 1982, Tolonen 1982), accumulate 22:1 FA to a lesser degree than do blue foxes (*Alopex lagopus*), which only consume fish occasionally if ever (Samuel and Nelson 1982), when both species were fed diets high in 22:1. The mink and seals studied here exhibited a strong capacity for the metabolism of 22:1n-11 as evidenced by the consistently lower recovery of ^{3}H 22:1n-11 relative to ^{14}C 18:1n-9.

In the mink incubated for 6 h after feeding labelled 22:1, the mesenteric adipose tissue showed the highest relative recovery of ^{3}H 22:1n-11 (Figure 2.2). Although the liver had the largest amount of ^{3}H 22:1 of any tissue analysed, among the adipose depots the mesenteric tissue contained the most ^{3}H 22:1 both in absolute and proportional terms (Table 2.1, Figure 2.3). These results were expected in light of what is known of the tissue distribution of peroxisomal β-oxidation activity. The intestinal wall can be an important site for the chain shortening of dietary FA (Thomassen *et al.* 1985), however, the liver is the main site of peroxisomal β-oxidation (Ong *et al.* 1977, Bremer and Norum

1982). Very little, if any, chain shortening is found in isolated adipocytes (Christiansen *et al.* 1979a). The mesenteric adipose tissue is the depot that has first access to ingested FA as they are transported from the small intestine via the lymph. Many of the FA found in this depot will not have passed through the liver prior to their deposition here, making the peroxisomal β-oxidation of the enterocytes the only chain-shortening activity to which they could have been exposed. A greater proportion of the FA deposited in the visceral and subcutaneous adipose depots will have cycled through the liver. This explains their much lower concentrations and relative recoveries of ^{3}H 22:1n-11 compared to the mesenteric adipose tissue.

In the 6 h mink, both the relative recovery of ^{3}H 22:1 and the distribution pattern of ^{3}H radioactivity across the various FA indicate that there is considerable individual variation in the ability to metabolize 22:1n-11. For example, Mink 3 appears to have a much higher capacity than either Mink 1 or 2 (Figures 2.2 and 2.3). This variation may be due to differences in the activity of their peroxisomal β-oxidation systems. It could also be caused by differences in food passage rate among the mink. The average food passage rate in mink is approximately 2-5 h (Joergensen 1985, Szymeczko and Skrede 1990, Atkinson 1996). After only a 6 h incubation period, each mink could have been at a different point in the processing of the meal. Consistent with this interpretation, there was much less individual variation in the same data from the 9 h mink. After a 9 h incubation period, the recovery of ^{3}H 22:1n-11 relative to ^{14}C 18:1n-9 was lower in all mink tissues studied and there was very little difference in the relative recovery of ^{3}H 22:1n-11 across depots (Figure 2.2). There was also a generally lower concentration of ^{3}H radioactivity in all tissues at the later sampling period (Table 2.1). The uniformity of

the relative recovery data and the lower ^{3}H concentrations and relative recoveries at 9 h suggest that the extra time provided by the 9 h incubation period allowed the mink to more fully metabolize the ^{3}H 22:1n-11.

The presence of ^{3}H in the chain-shortened products of 22:1, namely 20:1, 18:1 and 16:1, isolated from adipose samples of animals fed ^{3}H-labelled 22:1n-11 was consistent with previous studies (Figures 2.3-2.5). In peroxisomal chain shortening, only one or a few β-oxidation cycles take place (Osmundsen *et al.* 1979), making 20:1, 18:1 and 16:1 the expected products. Norseth and Christophersen (1978) found that the main product of the chain shortening of 22:1n-9 is 18:1n-9 with some 20:1n-9 and 16:1n-9 also being formed. Our results with 22:1n-11 are similar in that the proportion of total radioactivity found in 18:1 of all mink adipose depots, as well as seal blubber, was generally more than twice that found in either 16:1 or 20:1 (Figures 2.3-2.5).

The extent of ^{3}H radioactivity found in the SFA was somewhat surprising (Figure 2.3-2.5). Radioactivity can appear in these FA if the chain shortened products of peroxisomal β-oxidation are transported to the mitochondria for complete breakdown and the resultant acetyl groups are then utilized in *de novo* FA synthesis. The presence of the vast majority of the ^{3}H radioactivity of the liver in SFA shows that this process of recycling the ^{3}H-labelled acetyl units into *de novo* synthesized SFA is important in this organ. Whether the ^{3}H-labelled SFA present in the adipose depots originated in the depots themselves or were transported there from the liver is not known.

In both the visceral and subcutaneous adipose depots, the proportion of total radioactivity present in 18:1 was greater in the 9 h mink than it was in the 6 h mink (Figure 2.3 and 2.4). The small sample sizes and the cross-sectional nature of these data

prevent firm conclusions, but suggest that some of the *de novo* synthesized SFA may have been elongated and/or desaturated to form 18:1 FA. Isomers of individual FA cannot be isolated using reverse-phase HPLC so I cannot assess the contribution of the different isomers to the total ^{3}H radioactivity associated with individual FA at the two sampling times. The larger proportion of ^{3}H radioactivity associated with 18:1 at 9 h may simply be due to an increased amount of ^{3}H 18:1n-11 from continued chain shortening of the ingested ^{3}H 22:1n-11. If, however, it is caused by an increased contribution from the 18:1n-7 or 18:1n-9 isomers, this would further indicate a progression in the metabolism of the ingested radioactivity at this time.

Pinnipeds accustomed to consuming diets high in 22:1 FA are expected to have efficient peroxisomal chain-shortening systems. This expectation is supported by Iverson *et al.* (2004), who showed that the concentration of 22:1n-11 in the blubber of grey and harp (*Phoca groenlandica*) seals is much lower than its concentration in the diet (proportional recoveries of 0.20 and 0.34, respectively). We also found a reduced recovery of ^{3}H 22:1n-11, in this case relative to ^{14}C 18:1n-9, in the two seals studied here (0.66 and 0.84). The reduced deposition of 22:1n-11 calculated by Iverson *et al.* (2004) is measured in relation to all other FA present in the diet and blubber, including those that can be synthesised *de novo* in the seal. This ratio reflects total FA metabolism in the animal and, therefore, the ratio is likely lower than can be accounted for by the direct metabolism of 22:1n-11. Because the calculation of the recovery of 22:1n-11 is relative to only a single FA, 18:1n-9, this measure of reduced recovery more directly reflects the role of peroxisomal β-oxidation in determining the relationship between dietary and blubber levels of 22:1n-11.

The differences between the distribution patterns of ^{3}H radioactivity in the blubber of the two seals imply that Seal 1 had a higher capacity to metabolize the ingested ^{3}H 22:1n-11, both in terms of chain shortening and complete recycling into *de novo* synthesized FA (Figure 2.5). This suggestion is also supported by the lower relative recovery of ^{3}H 22:1n-11 in Seal 1 compared to Seal 2. Although the cause of this difference can only be speculated upon, Seal 1 may have had a higher mass specific metabolic rate, as a result of its smaller body size (42.0 kg vs. 50.5 kg), which could have lead to a more complete metabolism of the ingested ^{3}H 22:1n-11 at the time of sampling. Even though 22:1n-11 is a good indicator of diet and has proven to be an important FA when using adipose tissue FA composition to study diet (Iverson 1993, Kakela *et al.* 1993, Smith *et al.* 1996, Iverson *et al.* 1997a,b, Raclot *et al.* 1998, Brown *et al.* 1999, Dahl *et al.* 2000), this individual variation in the ability of animals to metabolize 22:1n-11, found in both mink and seals, could potentially reduce the usefulness of this FA in quantitative estimations of diet.

In the past, mink have been used as models for non-ruminant animals in general (Urlings *et al.* 1993) and carnivores specifically (Tauson *et al.* 1994). Because they are naturally carnivorous aquatic animals, mink are particularly reasonable model animals for studying marine mammals (Donnelly *et al.* 2000). My results indicate that mink are a good model to investigate the metabolism of marine lipids by a carnivore accustomed to their consumption. The distribution patterns of ^{3}H radioactivity across the FA isolated from seal blubber and mink subcutaneous adipose tissue were comparable (Figure 2.6). Mink have faster rates of passage of ingesta (2-5 h; Joergensen 1985, Szymeczko and Skrede 1990, Atkinson 1996) relative to seals (5-13 h; Helm 1984, Krockenberger and

Bryden 1994) as well as higher mass specific metabolic rates (Kleiber 1975), as a result of their smaller body size. Therefore, the 24 h incubation period used in the seal experiments appears to be roughly equivalent to the shorter incubation periods used in the mink experiments.

Chapter 3. Chylomicron fatty acid metabolism in grey seals

Introduction

Lipid metabolism can be separated into early and late stages. The early stages of lipid metabolism include the hydrolysis of FA from the ingested TAG, absorption of FA by the small intestine, and incorporation of FA into blood chylomicrons. Chylomicrons are TAG-rich lipoproteins synthesised in the small intestine, which act as the primary transport lipoproteins for dietary fat in the aqueous environment of the blood. They consist of a core of TAG and cholesterol esters surrounded by a PL monolayer with associated cholesterol and apolipoproteins (Zilversmit 1965). Although chylomicron TAG are not exclusively dietary in origin (Karmen *et al.* 1963, Sheehe *et al.* 1980, Mansbach and Parthasarathy 1982, Shiau *et al.* 1985) it is well documented that the FA composition of TAG in chylomicrons reflects that of the diet (Ockner *et al.* 1969, Griffiths *et al.* 1994, Lambert *et al.* 1996, Fielding *et al.* 1999, Summers *et al.* 2000). The FA composition of the other chylomicron lipids (PL and cholesterol esters), however, does not (Kayden *et al.* 1963, Whyte *et al.* 1963, Redgrave and Dunne 1975). Because TAG is the main class of FA-containing lipid in mammalian chylomicrons (80%-95%) (Redgrave 1983, Brindley 1991), the overall chylomicron FA composition is reflective of diet as well.

Differential metabolism of individual FA throughout digestion, absorption and incorporation into chylomicrons will influence the relationship between the FA composition of the diet and that in the adipose tissue. For example, in terrestrial species,

the *in vivo* partitioning of absorbed FA between newly synthesized PL and TAG has been shown to vary for individual FA (Nilsson *et al.* 1987a, Nilsson and Melin 1988 as cited in Nilsson *et al.* 1992, Perez *et al.* 1999). Because FA esterified in chylomicron TAG will be more readily incorporated into adipose TAG, if such partitioning of dietary FA between PL and TAG occurs in marine carnivores such as pinnipeds, it will affect the relationship between dietary and adipose tissue FA. Summers *et al.* (2000) suggest that this early metabolic processing of dietary FA is the main determinant of their deposition in human adipose tissue. Given the distinct characteristics of marine lipids and the expected adaptation of marine carnivores to consuming them, it is reasonable to predict that differences may exist between marine carnivores and terrestrial species in the relationship between dietary and chylomicron FA compositions. Few studies have looked at this relationship in marine carnivores. My first aim was, therefore, to investigate the metabolism of individual dietary FA at these early stages of assimilation by characterizing the FA composition of chylomicrons over the complete digestion period in grey seals and comparing these with that of the meal consumed.

My second aim was to investigate the capacity of chylomicron FA signatures to provide quantitative estimates of diet composition using QFASA (Iverson *et al.* 2004). One of the primary advantages of QFASA over other methods of diet analysis in pinnipeds is that it provides a relatively long-term integration of an animal's diet, as opposed to just its most recent intake. Situations exist, however, when the prey composition of a recent meal is of interest. If QFASA of chylomicron signatures is viable, it will provide another tool, which could be used in conjunction with longer-term QFASA diet estimates from adipose tissue of the same individual.

Methods

Animal Maintenance and Sampling

Six grey seals (age 2-3 yrs), originally captured as weaned pups from the Scotian Shelf population off eastern Canada, were housed in a seawater tank at the Dalhousie University Aquatron Facility. Seals had been maintained throughout on diets of various lots of Atlantic finfish species, but mostly Atlantic herring (*Clupea hargenus*). Prior to the start of our experiment, seals were fasted for approximately 24 h. Early the next morning, the seawater tank was drained and an initial blood sample (≤10 mL) was taken from the hind flipper of each seal into vacutainers containing anticoagulant. Each animal was then fed a meal from a single lot of Atlantic herring. Three seals received 2.3 kg of herring while the other three received 4.6 kg. Ten whole herring were randomly sampled from the same lot and stored in airtight plastic bags at –20°C until analysis. Seals were captured in a net at 1, 3, 6, 9, 12 and 24 h after feeding and a blood sample (≤ 10 mL) was taken at these times as above.

Chylomicron isolation

The lipoprotein classes of blood are defined and separated based on their densities. There is, however, some overlap in the sizes of the different classes. Chylomicrons are typically 35-250 nm in diameter, very low-density lipoproteins (VLDL) range from 30-110 nm, and the remaining lipoprotein classes are much smaller (Davis 1991). When a separation density of 1.006 g/mL is used, lipoproteins with diameters greater than 100 nm are isolated. I used this density so as to isolate the

majority of blood chylomicrons while minimizing contamination from VLDL. For all postprandial blood samples, I refer to this density fraction ($\rho < 1.006$ g/mL) as chylomicrons, despite some VLDL contamination. For the post-absorptive (e.g., 0 h and 24 h) plasma samples, VLDL will likely constitute a large proportion of the very small quantity of lipoproteins expected to be recovered. I, therefore, designate the isolated fraction here simply as post-absorptive lipoproteins.

Fresh blood samples were centrifuged and then visually inspected for the presence and relative amount of chylomicrons, as evidenced by the cloudy white layer floating at the top of the sample. Due to constraints placed on the captive sampling situation, which resulted in reduced volumes of blood being obtained as sequential blood sampling progressed, I was often unable to obtain sufficient sample for both accurate measurement of total lipid concentration and isolation and quantification of chylomicron FA. My primary interest was in accurately measuring chylomicron FA composition and linking this to visual appearance of their presence in blood samples, as this would be the situation faced by most investigators in the field when sampling free-ranging animals. Thus, blood samples were visually ranked as being clear or having low, moderate, high or very high concentrations of chylomicrons. Plasma (1-4 mL) was collected from these samples and overlaid with a solution of 0.196 M NaCl (ρ 1.006 g/ml), at pH 7.4 containing EDTA (0.01% w/v) and NaN_3 (2mM). The ratio of sample to saline was 1:1 v/v. The saline solution was carefully layered with a Pasteur pipette with the tip bent at a right angle and placed against the wall of the tube at the meniscus to avoid mixing. Tubes were then centrifuged at 26,000 g for 20 minutes at 15°C in a Spinco-Beckman L65 ultracentrifuge (Beckman, Palo Alto, CA, USA). The top layer, containing all lipoproteins with $\rho <$

1.006 g/mL, was carefully withdrawn, the centrifuge tube was refilled with saline and centrifuged again as described above. The lipoprotein fractions collected from the two centrifugations were pooled into a new centrifuge tube and 30-40 mg of polyethylene glycol (26,000 MW, BDH) were added. The same volume of saline as polyethylene glycol was added on top and the tubes were again centrifuged. The top layer, containing the purified chylomicrons or post-absorptive lipoproteins, was collected and frozen at -35°C in a N_2 atmosphere until further analysis.

Lipid Analysis

Lipids were extracted from the purified chylomicron samples into chloroform according to the method of Folch *et al.* (1957) as modified by Iverson (1988) using the ratios of 18 parts 2:1 choloroform-methanol (v/v) to 1 part sample and 3 parts solvent to 1 part aqueous salt. FAME were prepared from 100 mg of the pure extracted lipid (filtered and dried over anhydrous sodium sulfate) using 1.5 mL of 8% boron trifluoride in methanol (v/v) and 1.5 mL of hexane, capped under nitrogen, and heated at 100°C for 1 h (Morrison and Smith 1964). FAME were extracted into hexane, concentrated and brought up to volume (50mg/mL) with high-purity hexane.

Duplicate analyses of FAME were performed using temperature-programmed gas-liquid chromatography on a Perkin Elmer Autosystem II Capillary FID gas chromatograph fitted with a 30-m column (0.25-mm inside diameter) coated with 50% cyanopropyl polysiloxane (0.25-μm film thickness; JandW DB-23; Folsom, CA, USA). Identifications of FA were determined from a number of sources, including known

standard mixtures (Nu Check Prep., Elysian, MN, USA), silver-nitrate chromatography and GC-mass spectrometry (Iverson *et al.* 1992, 2002).

Data Analysis

In order to assess the degree to which chylomicron FA composition at the various sampling times resembled that of the test meal, the average Kulback-Leibler (K-L) distances (Kotz and Johnson 1983) between chylomicron and herring FA signatures were calculated. The average K-L distance represents a measure of the distance between two profiles of proportional data and is calculated as follows: $\Sigma_j (C_j - D_j)\log(C_j/D_j)$ where j = individual FA, C = one profile (e.g., chylomicron signature) and D = a second profile (e.g., test meal signature). The smaller the K-L distance the more similar are the two sets of proportional data. Significant differences in the levels of individual FA between the meal (n = 10) and chylomicron signatures sampled at the time corresponding to the lowest K-L distance (3 h post-feeding, n = 6) were then determined by a MANOVA that used the five (i.e., n-1) FA with the largest coefficient of variation.

Iverson *et al.* (2004) have developed a statistical model (QFASA) that provides quantitative estimates of the proportions of prey species in the diets of individual predators using FA signatures. I tested the model's ability to accurately identify the experimental herring as the only prey item in the meal, using the chylomicron FA signatures sampled at the various times post-feeding. All prey FA signatures used in this study, except that of the experimental meal, were taken from a FA database of prey collected from the Scotian Shelf, which consists of 954 individuals of 28 fish and invertebrate species (Budge *et al.* 2002). An important component of the QFASA model

is the use of calibration coefficients for individual FA to account for lipid metabolism within the predator. Calibration coefficients can be calculated when the complete diet FA signature that produced the predator FA signature is known. According to Iverson *et al.* (2004), the calibration coefficient, c_j, of a particular fatty acid (j) is computed as the 10% trimmed mean of the following r_{li}^{j}'s

$$r_{li}^{j} = seal_{ij}/diet_{lj}$$

for all l and i where l represents an individual diet signature and i represents an individual seal.

I used two sets of calibration coefficients to model the test meal of seals from their chylomicron signatures: 1) existing blubber calibration coefficients developed from a separate captive feeding study of grey seals (Iverson *et al.* 2004) and 2) chylomicron calibration coefficients calculated using the FA signatures of chylomicrons sampled at 3 h post-feeding (i.e., the lowest K-L distance from diet signature). Within each treatment group (2.3 kg meal and 4.6 kg meal), the chylomicron signatures of individual seals were modelled using the average calibration coefficients calculated from the other two seals in that group. I then modelled meals using two sets of prey inputs. The first set used only five potential prey options: argentine (*Argentina silus*), capelin (*Mallotus villosus*), northern sandlance (*Ammodytes dubius*), redfish (*Sebastes* sp.), and the herring actually fed to the seals in this study. These prey types were chosen based on hierarchical cluster analysis (Iverson *et al.* 2004), which showed that these prey FA signatures were more similar to each other than they were to all other species in the prey database, and thus represented a potentially difficult estimation scenario. The second prey set used the entire Scotian Shelf prey database and the experimental herring labelled separately from

the other herring in the database. Here I wished to determine whether an increased number of prey options introduced significant noise in the model predictions.

Results

I obtained sufficient lipid for chylomicron FA analyses in all 1, 3, 6 and 9 h postprandial samples. I was unable to obtain sufficient chylomicron lipid for reliable FA analysis in one 0 h post-absorptive sample, in four of the six 12 h samples and in all 24 h samples. This was in part due to very low lipid levels at these times, particularly 0 and 24 h (Figure 3.1), but primarily due to the reduced volumes of blood obtained as sequential sampling progressed (especially at 12 and 24 h, see Methods). The qualitative visual assessment of serum samples for the presence and relative amount of chylomicrons (cloudy white layer) indicated that chylomicrons began to appear in the blood by 1 h post-feeding (Figure 3.1). The relative concentration of chylomicrons in the blood peaked between 3-6 h post-feeding and then slowly declined until chylomicrons were no longer evident at some point between 12 and 24 h post-feeding. All study animals exhibited this pattern, however, the concentrations of chylomicrons in seals fed the 4.6 kg meal appeared to peak earlier, were generally greater, and persisted longer than those of the seals fed the 2.3 kg meal.

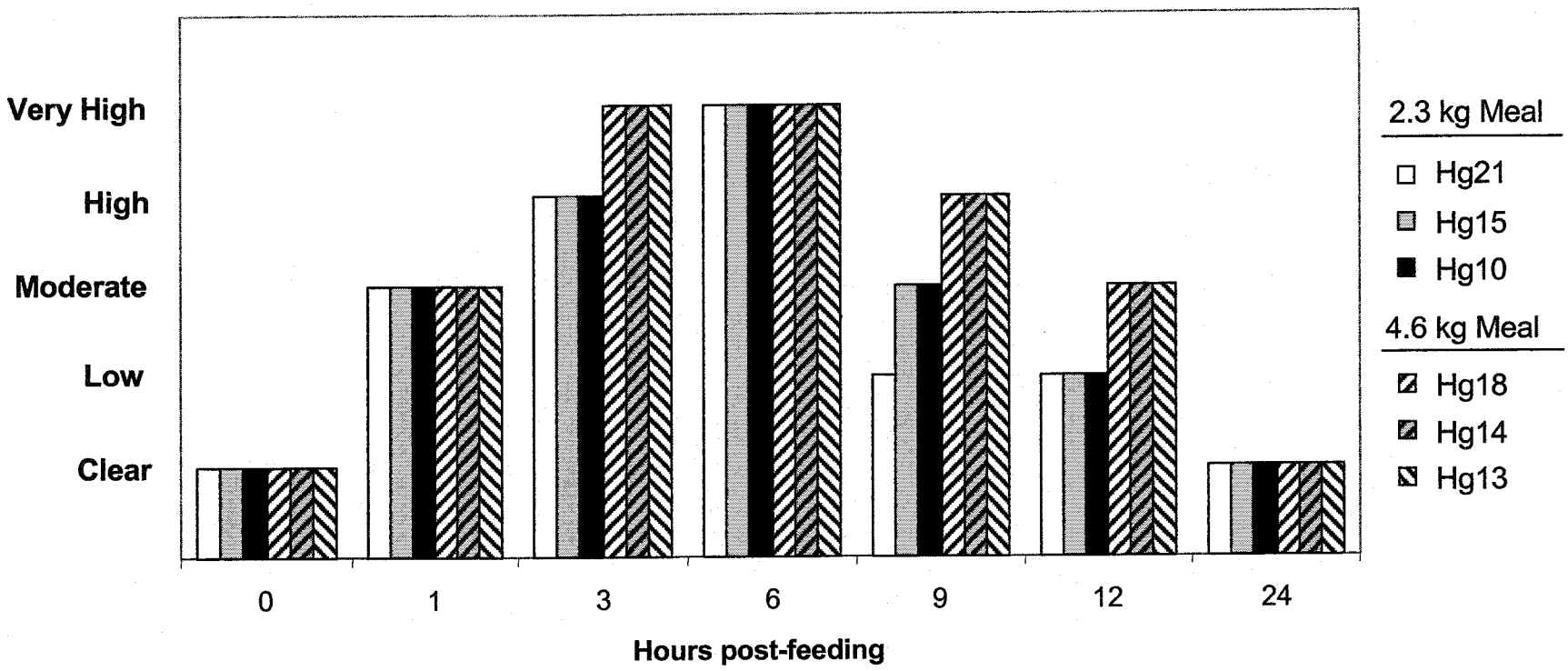

Figure 3.1: Appearance and visual assessment of chylomicron concentration in serum samples taken at various times post-feeding. *Solid bars*, seals fed 2.3 kg of experimental meal. *Hatched bars*, seals fed 4.6 kg of experimental meal.

The lowest K-L distances calculated between the test meal and chylomicron FA signatures occurred at 3 h post-feeding, indicating that the FA signatures of chylomicrons and the meal were most similar at this time. Values did not differ between meal sizes at this time but all other distances were lower for seals fed the 4.6 kg meal than for those fed the 2.3 kg meal (Figure 3.2), indicating greater similarity with increased meal size (i.e., fat intake). Both groups exhibited similar temporal patterns in K-L distances over time, with the greatest distances measured in the essentially post-absorptive lipoprotein samples (0 h and12 h).

The FA composition of the herring meal, in comparison to that of the post-absorptive lipoproteins (0 h) and postprandial chylomicrons (3 h), is presented in Table 3.1. The herring diet contained 19% SFA, 62% MUFA, and 19% PUFA. The majority of each of these classes was accounted for by only two FA each: 90% of the SFA were 14:0 and 16:0, 69% of the MUFA were 20:1n-9 and 22:1n-11, and 60% of the PUFA present were 20:5n-3 and 22:6n-3. The dominant FA found in the seals' post-absorptive lipoproteins were generally those that were dominant in the test-herring, which also represented the habitual diet. However, the relative proportions of total SFA, MUFA and PUFA (28%, 42% and 31%, respectively) in post-absorptive lipoproteins differed considerably from those in herring, as did the relative contributions to these classes of individual FA (Table 3.1). For example, these lipoproteins were extremely enriched in18:0, 18:2n-6, and 20:4n-6, and reduced in 20:1n-9 and 22:1n-11 relative to the diet. In contrast to these post-absorptive lipids, the FA composition of the 3 h postprandial chylomicrons was similar to that of the meal, with SFA, MUFA and PUFA comprising about 20%, 54% and 26% of total FA, respectively (Table 3.1). Nevertheless, some

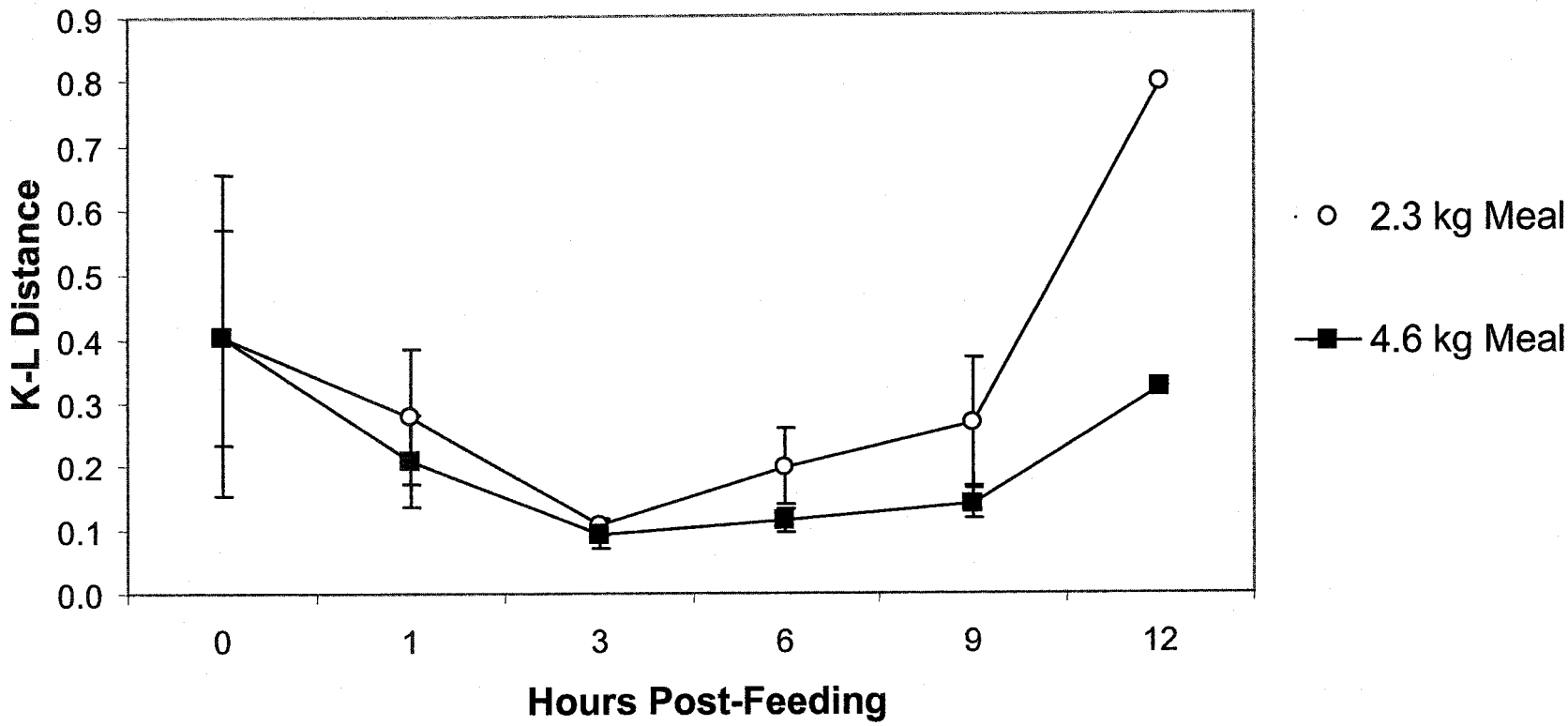

Figure 3.2: Average Kulback-Leibler distance (± 1 SD) between the FA signature of the meal and those of the chylomicrons sampled at various times post-feeding; sample sizes for each group are n = 3 except at 0 h (n = 2, for 2.3 kg group) and 12 h (n = 1 for each group) samplings.

Table 3.1: Fatty acid composition of the herring diet in comparison to post-absorptive (0 h) lipoproteins and post-prandial (3 h) chylomicrons from seals fed either 2.3 or 4.6 g of the diet. Values are mean mass percent ± SD of fatty acids (35 out of 69) present at levels 0.2%. Sufficient lipid for analysis was only obtained for 5 of the 6 seals at 0 h.

			Chylomicrons	
Fatty Acid	Herring Diet	0h	3h 2.3 kg	3h 4.6 kg
	n = 10	n = 5	n = 3	n = 3
14:0	6.4 ± 0.39	3.6 ± 1.19	4.9 ± 0.20	4.6 ± 0.14
14:1n-9	0.2 ± 0.01	0.4 ± 0.15	0.5 ± 0.01	0.5 ± 0.06
15:0	0.3 ± 0.01	0.4 ± 0.06	0.3 ± 0.02	0.3 ± 0.03
16:0	10.6 ± 0.31	13.8 ± 1.68	10.8 ± 0.58	10.7 ± 0.55
16:1n-11	0.3 ± 0.02	0.7 ± 0.27	0.5 ± 0.06	0.5 ± 0.02
16:1n-9	0.1 ± 0.01	0.3 ± 0.15	0.2 ± 0.06	0.2 ± 0.03
16:1n-7	5.3 ± 0.39	3.1 ± 1.10	4.3 ± 0.29	4.4 ± 0.13
16:2n-6	0.1 ± 0.01	0.5 ± 0.11	0.3 ± 0.02	0.5 ± 0.09
16:3n-6	0.7 ± 0.03	0.4 ± 0.23	0.4 ± 0.01	0.4 ± 0.00
16:3n-4	0.4 ± 0.04	0.4 ± 0.18	0.4 ± 0.06	0.3 ± 0.02
16:4n-1	0.8 ± 0.09	0.6 ± 0.20	0.7 ± 0.06	0.4 ± 0.17
18:0	0.9 ± 0.05	6.7 ± 3.01	2.7 ± 0.16	2.8 ± 0.60
18:1n-11	0.6 ± 0.04	2.0 ± 0.69	1.0 ± 0.09	1.1 ± 0.08
18:1n-9	5.7 ± 0.91	9.3 ± 2.82	6.4 ± 0.03	6.4 ± 0.25
18:1n-7	1.6 ± 0.13	2.6 ± 0.58	1.9 ± 0.10	1.9 ± 0.13
18:1n-5	0.4 ± 0.02	0.3 ± 0.07	0.4 ± 0.06	0.4 ± 0.02
18:2n-6	0.8 ± 0.05	2.8 ± 1.70	1.0 ± 0.09	1.2 ± 0.14
18:3n-3	0.5 ± 0.08	0.4 ± 0.12	0.5 ± 0.05	0.5 ± 0.04
18:4n-3	1.6 ± 0.33	1.0 ± 0.48	1.3 ± 0.12	1.4 ± 0.05
20:0	0.2 ± 0.01	0.6 ± 0.18	0.3 ± 0.02	0.3 ± 0.01
20:1n-11	1.0 ± 0.08	1.4 ± 0.16	1.4 ± 0.03	1.3 ± 0.03
20:1n-9	16.7 ± 0.36	7.9 ± 3.56	12.7 ± 0.71	12.0 ± 0.44
20:1n-7	0.5 ± 0.12	0.3 ± 0.07	0.6 ± 0.04	0.5 ± 0.03
20:4n-6	0.2 ± 0.01	3.3 ± 1.36	1.6 ± 0.11	1.5 ± 0.18
20:4n-3	0.3 ± 0.03	0.8 ± 0.16	0.5 ± 0.04	0.5 ± 0.06
20:5n-3	6.1 ± 0.42	10.1 ± 2.09	10.2 ± 0.54	10.9 ± 1.07
22:1n-11	25.7 ± 0.60	8.9 ± 6.07	20.0 ± 1.58	18.5 ± 1.23
22:1n-9	1.7 ± 0.24	1.9 ± 0.68	1.5 ± 0.17	1.6 ± 0.17
22:1n-7	0.3 ± 0.05	0.2 ± 0.06	0.3 ± 0.03	0.4 ± 0.11
21:5n-3	0.2 ± 0.03	0.2 ± 0.08	0.3 ± 0.01	0.3 ± 0.03
22:5n-6	0.1 ± 0.00	0.3 ± 0.26	0.3 ± 0.13	0.4 ± 0.14
22:5n-3	0.7 ± 0.03	1.5 ± 0.39	1.1 ± 0.03	1.0 ± 0.06
22:6n-3	5.5 ± 0.45	5.0 ± 1.27	5.5 ± 0.50	5.5 ± 0.39
24:1n-11	0.3 ± 0.02	0.3 ± 0.22	0.3 ± 0.09	0.5 ± 0.15
24:1n-9	0.7 ± 0.04	1.0 ± 0.26	0.9 ± 0.06	0.9 ± 0.07
Saturated	19.2 ± 0.71	27.5 ± 4.43	20.3 ± 0.81	20.5 ± 1.55
Monounsaturated	61.6 ± 1.83	41.7 ± 6.36	53.6 ± 2.27	52.2 ± 1.11
Polyunsaturated	19.2 ± 1.33	30.8 ± 3.18	26.1 ± 1.46	27.3 ± 0.45

differences remained between the absolute levels of individual FA in chylomicrons and those in the diet. For instance, levels of 18:0, 20:4n-6 and 20:5n-3 were higher while 20:1n-9 and 22:1n-11 were lower in chylomicrons compared to diet.

Although I used the calibration coefficients calculated from the 3 h chylomicrons for the actual diet modeling, I calculated coefficients from all post-feeding chylomicron signatures to assess the degree to which these differed from one another (Figure 3.3). The majority of FA whose ratio deviated greatly from 1.0 were present in very small amounts (<0.5%). The exceptions to this were 20:1n-9 and 22:1n-11 which had ratios significantly less than 1.0, and 18:0, 20:4n-6 and 20:5n-3 which had ratios significantly greater than 1.0 (MANOVA, $p<0.001$). The ratios between chylomicron and diet FA also changed over time. Consistent with the findings from K-L distances, ratios generally had the smallest deviations from 1.0 at 3 h post-feeding and subsequently increased in the later postprandial samples, with the greatest differences observed in 12 h chylomicrons. I compared the 3 h chylomicron coefficients with those previously calculated from grey seal blubber signatures (Iverson *et al.* 2004) (Figure 3.4). The calibration coefficients of the FA that were greater in chylomicrons compared to blubber included 20:4n-6, all *iso* FAs, 18:2Δ5,11, the SFA 18:0, and 20:0, and the isomers of 22:1. The FA with higher calibration coefficients in blubber compared to chylomicrons were 22:5n-3 and the MUFA 14:1n-5, 17:1, 20:1n-11, and all isomers of 16:1 and 18:1.

The QFASA model accurately identified the experimental herring as the main prey item contributing to the chylomicron signatures (and thus meal), using the reduced prey set of similar signatures and either the blubber or chylomicron calibration coefficients (Figures 3.5a and 3.5b). At 3 h and 6 h post-feeding, when chylomicrons

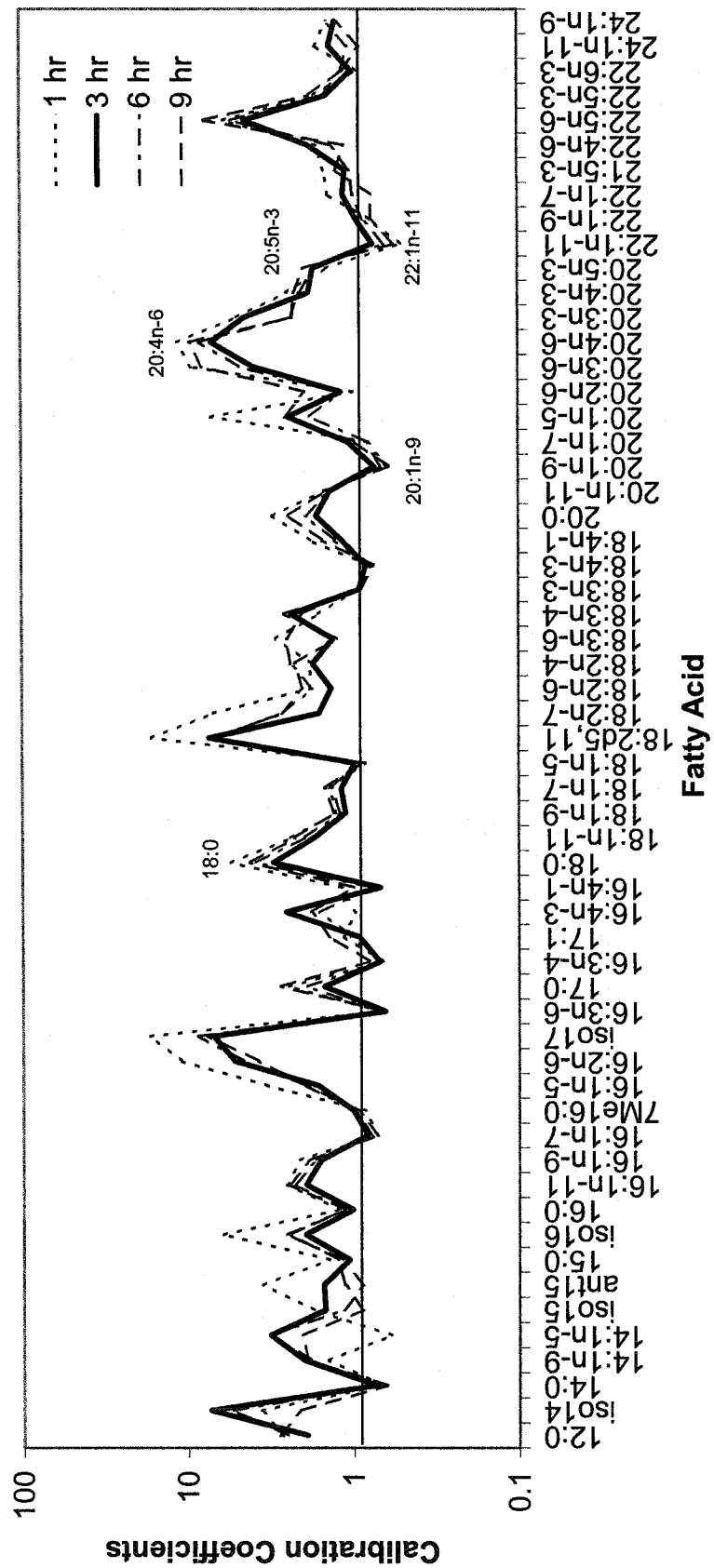

Figure 3.3: Log plot of calibration coefficients for individual FA calculated from the average chylomicron FA signatures (2.3 and 4.6 kg meals combined) measured at the various times post-feeding. A value > 1.0 indicates that the FA is present at a higher concentration in the chylomicrons than in the meal and visa versa for a value < 1.0.

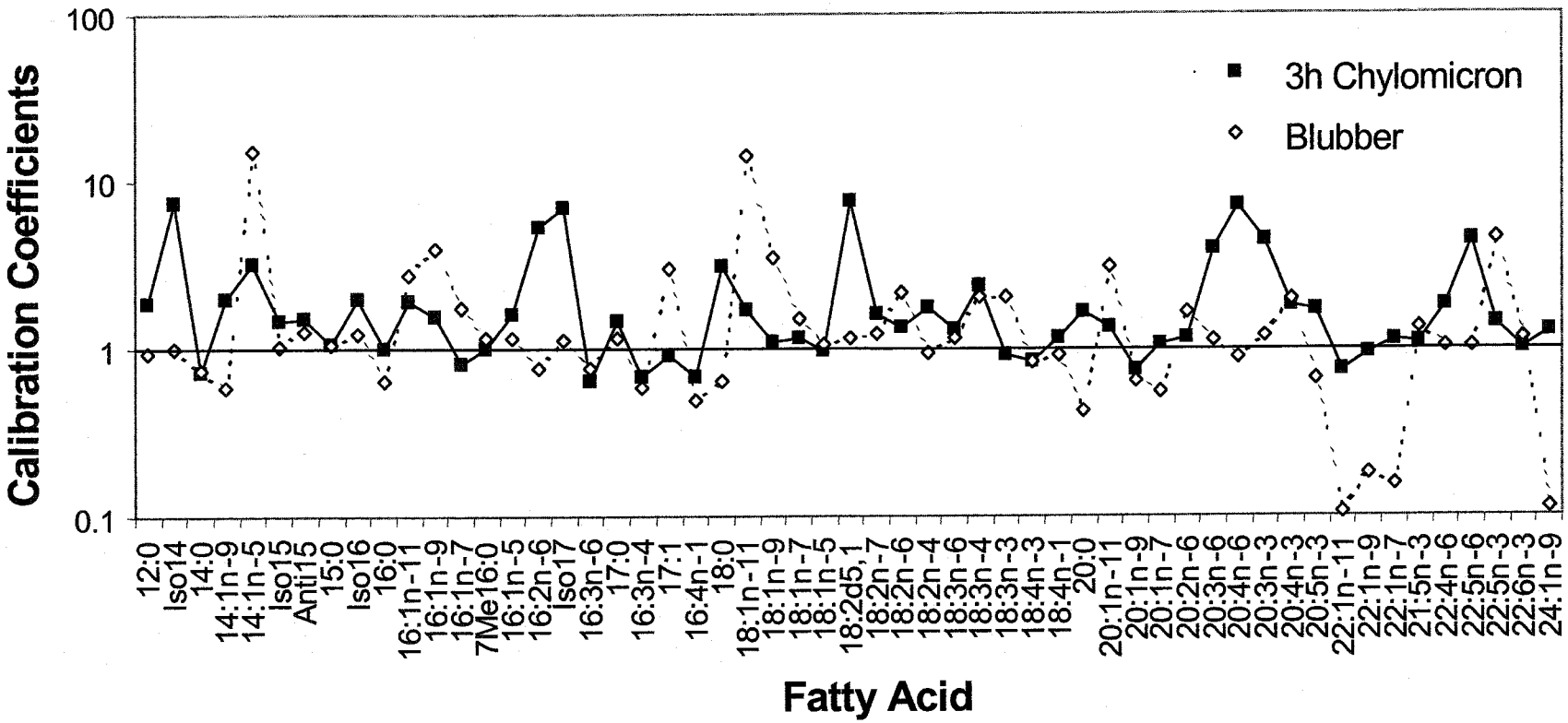

Figure 3.4: Log plot of the calibration coefficients calculated from chylomicron FA signatures measured at 3 h post-feeding (2.3 and 4.6 kg meals combined) in comparison to those previously reported (Iverson et al., 2004) from grey seal blubber FA signatures. A value > 1.0 indicates that the FA is present at a higher concentration in the chylomicrons or blubber than in the meal and visa versa for a value < 1.0.

were most abundant in all animals (Figure 3.1), the model estimated the experimental herring to comprise 90-100% and 75-100% of the meal, respectively. Estimates were generally less accurate using either the 1 h or 9 h chylomicrons and degraded entirely using the 12 h chylomicrons, especially with the animals fed 2.3 kg herring. The meal was generally best estimated using the blubber coefficients and in the animals fed the 4.6 kg meal. Sandlance and redfish were the main other prey species estimated as contributing to the chylomicron signatures, particularly at 12 h post-feeding.

When chylomicron signatures were modelled using the same calibration coefficients but with the entire Scotian Shelf prey database, the diet was again extremely well estimated (Figures 3.6a and 3.6b). The experimental herring was estimated to comprise about 100% of the meal at the 3 h sampling with either meal size, and >90% of the meal at both 1 h and 6 h post-feeding. Again, the estimates degraded in the 9 h and especially 12 h samples, particularly in the animals fed the smaller meal size. At all times, the experimental herring was identified as the herring source rather than the other samples of herring contained in the Scotian Shelf prey database. Sandlance, redfish, lobster (*Homarus americanus*) and rock crab (*Cancer irroratus*) were the main prey items erroneously estimated as contributors to the meal, especially at 12 h post-feeding.

Figure 3.5: Model estimates of the percent contribution of prey items to the meal using chylomicron FA signatures at the various times post-feeding and (a) calibration coefficients derived from blubber FA signatures (Iverson et al., 2004) or (b) calibration coefficients derived from 3 h chylomicron signatures. Meals were modeled with a prey input of the experimental herring and four species from the Scotian Shelf prey database (Budge et al., 2002) with relatively similar FA signatures to that of herring to assess a potentially difficult estimation scenario.

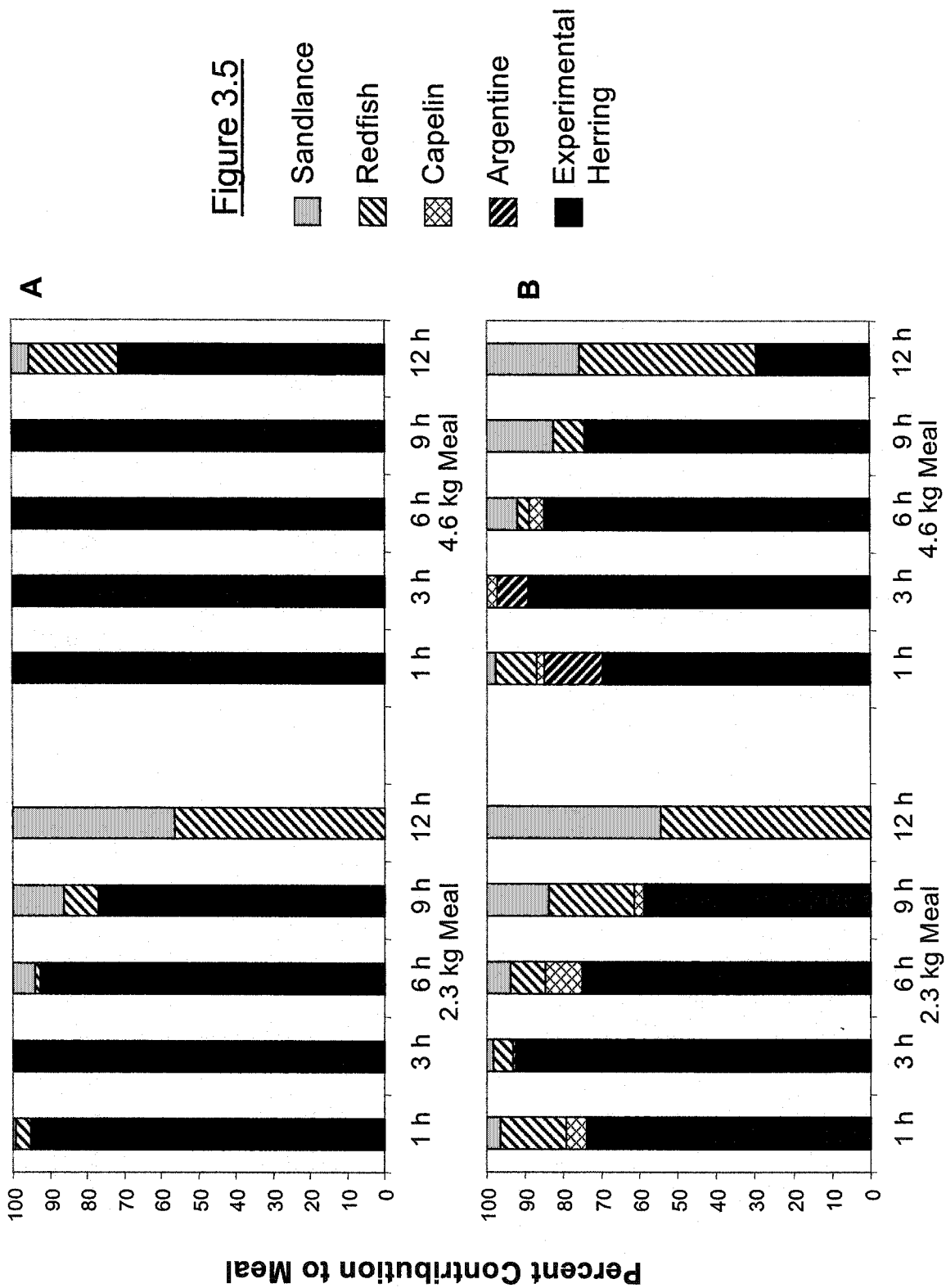
Figure 3.5
Sandlance
Redfish
Capelin
Argentine
Experimental Herring
A
B
Percent Contribution to Meal
100
90
80
70
60
50
40
30
20
10
0
1 h
3 h
6 h
9 h
12 h
1 h
3 h
6 h
9 h
12 h
2.3 kg Meal
4.6 kg Meal

Figure 3.6: Model estimates of the percent contribution of prey to the meal using chylomicron FA signatures at the various times post-feeding and (a) calibration coefficients derived from blubber FA signatures (Iverson et al., 2004) or (b) calibration coefficients derived from 3 h chylomicron signatures. Meals were modeled with a prey input of the experimental herring and the entire Scotian Shelf prey database of 28 species, including a different lot of herring (Budge et al., 2002). Only the prey species that appeared in any of the meal estimates are listed on the figure.

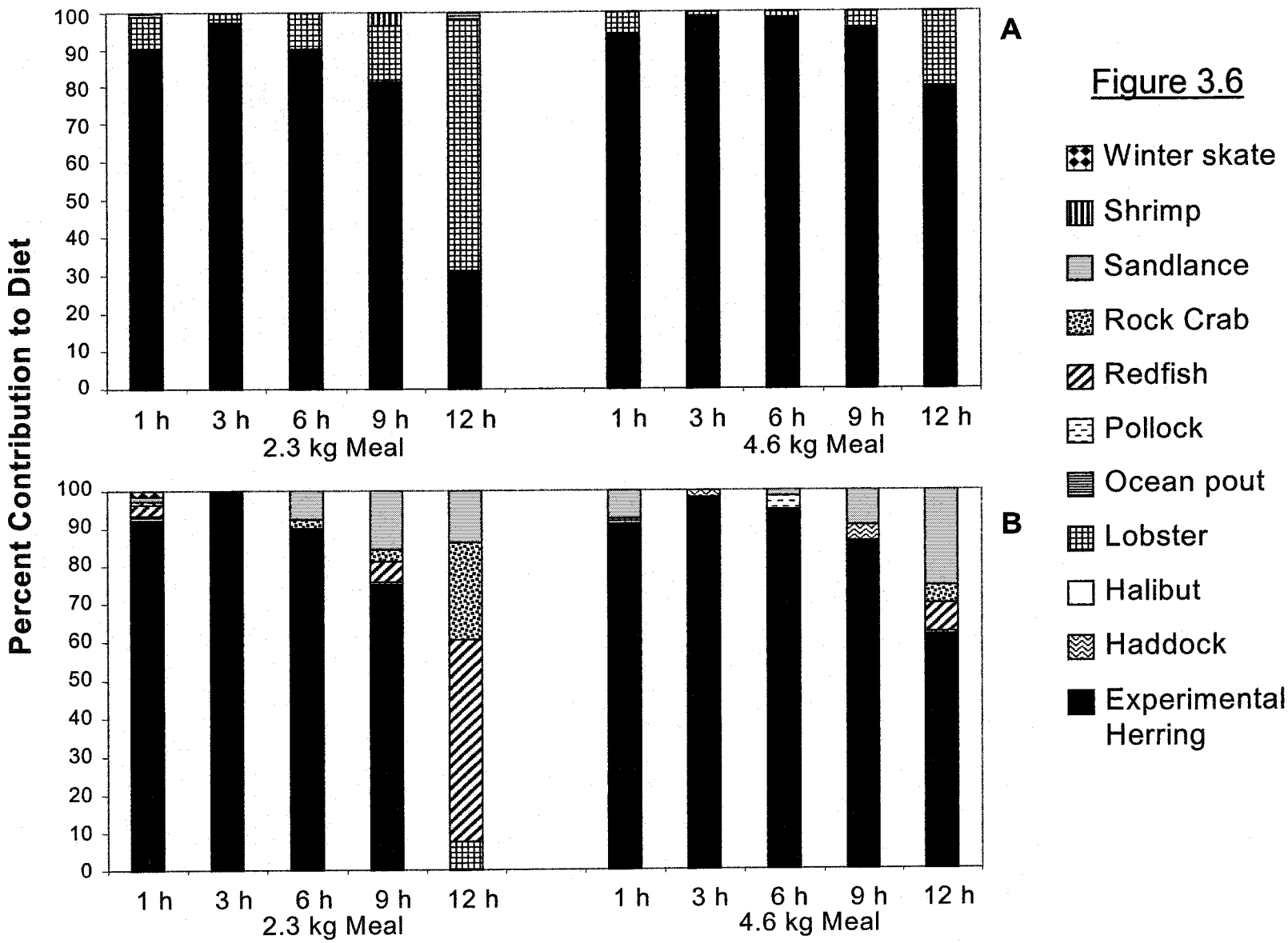
Percent Contribution to Diet
100
90
80
70
60
50
40
30
20
10
0
1 h
3 h
6 h
9 h
12 h
2.3 kg Meal
4.6 kg Meal
A
B
Winter skate
Shrimp
Sandlance
Rock Crab
Redfish
Pollock
Ocean pout
Lobster
Halibut
Haddock
Experimental Herring

Figure 3.6

Discussion

The results of this study demonstrate that chylomicron FA signatures resemble those of diet, but more importantly can be used to make accurate, quantitative predictions of the prey composition of a recent meal in a marine carnivore. Nevertheless, I have also shown that individual dietary FA likely experience differential metabolism leading up to their incorporation into chylomicrons. These differences should ultimately have an effect on the incorporation of different FA into predator adipose tissue. Therefore, studying the metabolism of individual FA at these early stages is important for understanding the relationship between dietary and adipose tissue FA compositions. When the differences between the FA composition of whole chylomicrons and that of the diet are accounted for by using calibration coefficients for individual FA, the QFASA model of Iverson *et al.* (2004) accurately predicts the meal when postprandial chylomicrons are present. The model identified the specific lot of herring fed over all other lots of herring, as well as other prey, contained in the broader Atlantic prey database. Although further work should examine mixed-species diets, this is the first time that diet has been studied, in a quantitative fashion, using the FA composition of chylomicrons.

Fatty acid metabolism within the predator

In this study, I was not able to accurately measure total lipid concentration of serum samples and, therefore, used the visual assessment of serum samples as an index for the presence and relative concentration of chylomicrons. Since TAG account for the majority of FA in chylomicrons (Redgrave 1983, Brindley 1991), I did not separate

chylomicron TAG and PL prior to measuring chylomicron FA composition. However, the degree to which PL FA contribute to the overall chylomicron FA composition clearly depends on both the time since feeding and the level of fat consumed, and is discussed below.

The temporal appearance of chylomicrons in grey seals (Figure 3.1) agrees well with studies performed on humans and rats in which chylomicrons typically appeared at some point before 2 h post-feeding and chylomicron TAG concentration peaked between 4-6 h post-feeding (Harris *et al.* 1988, Gibney and Daly 1994, Griffiths *et al.* 1994, Sakr *et al.* 1997, Lai and Ney 1998, Summers *et al.* 2000). The finding that the FA profiles of the chylomicrons sampled at 3 h post-feeding were most similar to that of the diet (i.e. smallest K-L distance, Figure 3.2) is also in keeping with this. While the density gradient used for isolating chylomicrons was aimed at minimizing contamination from VLDL, it is possible that as the amount of chylomicrons decreased with time post-feeding the relative contribution of VLDL FA to the overall signature increased. This could, in turn, increase the K-L distance between the chylomicron and diet signatures. Chylomicrons were still visible in all study animals at 12 h post-feeding (Figure 3.1). Lai and Ney (1998) found that, in rats, chylomicron TAG concentration remained above fasting values for at least 12 h post-feeding. In humans, however, Sakr *et al.* (1997) found that by 8 h post-feeding the concentration of the chylomicron fraction was at or below fasting values.

The earlier apparent peak and generally greater concentration of chylomicrons in the seals fed 4.6 kg of herring relative to those fed 2.3 kg (Figure 3.1) was expected. The former seals consumed twice as much fat and would, therefore, have more to transport in the blood. Chylomicron size is primarily dependent on the amount of TAG absorbed and

transported (Fraser *et al.* 1968, Boquillon *et al.* 1977, Hayashi *et al.* 1990). As the fat load increases, the size but not number of chylomicrons also increases. Larger chylomicrons have higher TAG:PL ratios and as a result their FA composition should be more reflective of diet. The finding that the K-L distances were consistently lower for seals fed the larger meal (Figure 3.2) is in keeping with their greater fat intake and suggests that these seals may have been transporting relatively larger chylomicrons, with a higher TAG:PL ratio.

The FA that were present in greater levels in the seal chylomicrons relative to their experimental diet have to have arisen from endogenous sources. These results are supported by several other studies that have also found relatively large endogenous contributions to the levels of certain FA in chylomicrons (Christensen and Høy 1996, Lambert *et al.* 1996, Becker *et al.* 2001). The primary endogenous sources of mucosal FA are plasma non-esterified FA and lipoproteins (Gangl and Ockner 1975, Gangl and Renner 1978, Mansbach and Dowell 1992), chylomicron remnants (Mansbach and Dowell 1994, 1995), and bile (Noma 1964, Shrivastava *et al.* 1967, Melin *et al.* 1996). The differential handling of individual FA, both in terms of chylomicron formation as well as during subsequent lipoprotein metabolism, determines the FA composition of endogenous sources. This, in turn, influences the FA composition of future chylomicrons. Because chylomicrons preferentially incorporate nascent TAG and preformed PL (Mansbach and Parthasarathy 1982, Mansbach and Nevin 1998, Luchoomun and Hussain 1999), it is likely that a proportionately greater amount of the endogenous FA found in the whole chylomicrons are associated with the PL fraction.

The partitioning of dietary FA between PL and TAG within the enterocyte differs for individual FA. In previous studies, 18:0, 20:4n-6 and 20:5n-3 all show greater incorporation into PL than do other FA (Whyte *et al.* 1963, Chen *et al.* 1985, Nilsson *et al.* 1987a, Nilsson and Melin 1988, Nilsson *et al.* 1992, Emken *et al.* 1993, Perez *et al.* 1999). Subsequent lipoprotein metabolism then contributes to the ultimate return of these FA to the intestine via endogenous sources, where they are again likely to become associated with the PL portion of the chylomicrons. The action of lipoprotein lipase collapses the chylomicron, resulting in the budding of some PL from the surface to form high-density lipoproteins (Redgrave and Small 1979) with the remaining lipids constituting the chylomicron remnant. These high-density lipoprotein PL can then be recycled through the liver with some eventually returning to the intestinal cells for incorporation into future chylomicrons (Fielding and Fielding 1991). Chylomicron and VLDL TAG-associated 20:4n-6, 20:5n-3, and 22:6n-3 have been shown to be relatively resistant to hydrolysis by lipoprotein lipase (Ridgway and Dolphin 1984, Nilsson *et al.* 1987b, Ekström 1989, Melin *et al.* 1991, Levy and Herzberg 1999). Wang and Koo (1993) showed that 18:0 is also released slowly from chylomicrons. For this reason, chylomicron remnants are generally enriched in these FA relative to their parent chylomicrons (Nilsson and Landin 1988, Melin *et al.* 1991, Nilsson *et al.* 1992, Mansbach and Dowell 1995, Lambert *et al.* 1996, Hansen *et al.* 1998). In keeping with these findings, three of the principle FA that exhibited significantly higher levels in grey seal chylomicrons than in the diet were 18:0, 20:4n-6 and 20:5n-3 (Table 3.1; Figure 3.3). Given that the relative enrichment of these FA increased in chylomicrons as time since feeding increased and were highest in post-absorptive lipoproteins, which would have

had higher PL to TAG ratios, I suggest that these FA were likely preferentially associated with the PL fraction of lipoproteins.

FA that get recycled through the liver also have the potential of returning to the intestinal cells via their presence in and absorption from bile. Rat bile PL can contain 15-20% 20:4n-6 (Kawamoto *et al.* 1980, Patton *et al.* 1984). Both 20:4n-6 and 20:5n-3 have high affinities for pathways that catalyze their incorporation into tissue PL (Pelech and Vance 1984, Leyton *et al.* 1987, MacDonald and Sprecher 1991) suggesting that when the habitual diet contains significant quantities of 20:5n-3 this FA would also be predominant in bile. However, Levy and Herzberg (1996) studied the FA composition of bile PL in rats adapted to fish oil diets and found that even when diets containing 13% 20:5n-3 were consumed this FA comprised less than 2% of the bile PL FA. Thus, bile PL may be an endogenous source of 20:4n-6 in grey seals but this may not be the case for 20:5n-3.

The experimental diet consisted primarily of MUFA, as did the 3 h chylomicrons but to a somewhat lesser degree (Table 3.1). This was mainly the result of lower levels of 20:1n-9 and 22:1n-11 in the chylomicrons relative to the diet (Table 3.1, Figure 3.3). These lower concentrations could in part result from dilution of dietary FA with the endogenous FA of the PL fraction. However, it is likely that they are primarily due to the partial chain shortening of these FA, via peroxisomal β-oxidation, within the enterocytes (Novikoff and Novikoff 1972, Thomassen *et al.* 1985). The peroxisomal β-oxidation system is quite active with 20:1 and 22:1 fatty acyl-CoA esters as substrate (Osmundsen *et al.* 1979) and is induced by the intake of 22:1 FA-containing diets (Christiansen *et al.* 1979, Neat *et al.* 1980, Neat *et al.* 1981, Thomassen *et al.* 1985, Veerkamp and

Zevenbergen 1986, Rouvinen and Kiiskinen 1989). Considering that my study animals had been maintained on a diet of Atlantic herring, which contains a high concentration of 22:1n-11 (26%, Table 1), their peroxisomal β-oxidation systems were expected to be highly active. This assumption was supported by the finding that the levels of 20:1n-9 and 22:1n-11 in chylomicrons were at most only 74% and 75%, respectively, of that in the diet (Table 3.1). Further support for this conclusion comes from other FA that were somewhat enriched in chylomicrons relative to diet. In peroxisomes only one or a few β-oxidation cycles take place. Thus the main products of the chain shortening of 22:1n-11 are thought to be 20:1n-11 and 18:1n-11. Both of these FA were present in greater quantity in the seal chylomicron FA than they were in the diet (1.3 and 1.8-fold respectively, Figure 3.3). It is difficult to assess the extent of chain shortening of 20:1n-9 by looking at the concentration of 18:1n-9 in the chylomicrons, as 18:1n-9 was present in significant quantities in the diet (6 %, Table 3.1) and there are several other factors influencing its concentration in chylomicron lipids.

The differences between calibration coefficients calculated using chylomicron signatures versus blubber signatures are extremely informative. Because the dietary FA concentration is always in the denominator of the calculation, these differences in calibration coefficients are indicative of differences between the levels of individual FA in the two fat sources, one immediately after absorption and one after final deposition in tissue. This, in turn, provides information regarding the origin of certain FA as well as the predominant location of various modification processes. For example, the calibration coefficient for 22:1n-11 was much lower for blubber than for chylomicrons, whereas those for 20:1n-11, 18:1n-11 and 16:1n-11 were much higher. This suggests that despite

the fact that some chain-shortening in the mucosa was implied by the chylomicron composition (Figure 3.3), the primary site of peroxisomal β-oxidation is downstream of chylomicron formation and most likely resides in the liver as has been suggested previously (Ong *et al.* 1977, Bremer and Norum 1982).

Differences between chylomicron and blubber calibration coefficients of several other FA are also in keeping with the fact that the majority of FA modification occurs post chylomicron formation (Cook 1991). For instance, the calibration coefficient of 22:5n-3, which is a modification product of 20:5n-3 (Terano *et al.* 1983, von Schacky and Weber 1985), was greater in blubber than in the chylomicrons, implying that the primary site of this inter-conversion reaction may reside in either the liver or the blubber itself. Secondly, the calibration coefficients of SFA were typically higher in the chylomicrons than in the blubber whereas the calibration coefficients of their immediate desaturation products were higher in the blubber. In a comparison between FA concentrations of chylomicrons and depot fat, Becker *et al.* (2001) found identical patterns in rats. Finally, the seal chylomicron calibration coefficients for the branched chain FA (*i*14:0, *i*15:0, *i*16:0 and *i*17:0) were > 1.0 and relatively larger for chylomicrons than for blubber (Figure 3.3 and 3.4). This is in keeping with both their proposed origin and site of metabolism. High proportions of branched chain FA have been linked to several categories of gut bacteria including Gram positive micro-organisms and certain Gram negative anaerobes (Moore *et al.* 1994, Hopkins *et al.* 2001), which accounts for their enrichment in chylomicrons. The relatively higher levels of these FA in chylomicrons compared to blubber, is likely due to their subsequent metabolism within the predator. Branched-chain FA are primarily oxidized within the peroxisomes (Poulos

et al. 1988, Singh *et al.* 1992). As discussed above, peroxisomal β-oxidation in my study animals was likely very active. As a result, the branched chain FA present in chylomicrons would be effectively oxidized, primarily in the liver, and thus not available for deposition in the blubber.

Quantitative diet estimation from chylomicron fatty acids

The QFASA model preformed well in estimating diets from postprandial chylomicron signatures both when using a select group of prey items of similar FA composition (Figure 3.5) and when using the entire Scotian Shelf prey base (Figure 3.6). In fact, when the chylomicron calibration coefficients were used, the model produced more accurate predictions when the entire prey base was included as opposed to just the select group of similar prey items (Figures 3.5b and 3.6b). In the former case, the model identified the experimental herring as the primary diet item over all other prey (28 species) in the database, including other lots of herring. Nevertheless, some prey species were falsely identified as contributing minor amounts to the meal. The appearance of redfish and sandlance with both prey sets, especially at 9 and 12 h post-feeding (Figures 3.5 and 3.6), was likely due to the similarity of their FA signatures to that of herring and the increased "noise" introduced in chylomicron signatures as the PL:TAG ratios increased with time since feeding. The appearance of crustaceans in the model predictions using the full prey base (Figure 3.6), again especially at 9 and 12 h post-feeding, was due to the very high levels of n-6 FA, particularly 20:4n-6, in these prey. The model included these prey in the estimates to account for the level of 20:4n-6 being much higher in the seal chylomicron signatures than in the diet.

Although the QFASA model provided accurate estimations of diet in postprandial chylomicrons, the time since feeding and the size of the meal influenced the accuracy of the results. The predictions were best when 3 h chylomicron signatures were used. When chylomicrons sampled within 9 h of feeding were used, predictions were still generally better than 75% and 85% accurate for seals fed 2.3 kg or 4.6 kg of herring, respectively. However, estimates clearly degrade as the time post-feeding increases. The processes involved in the metabolism of chylomicrons that increase the ratio of PL to TAG with time undoubtedly contribute to this finding. Isolation of TAG-FA from PL-FA in the samples taken within 9 h of feeding would, therefore, have improved my diet estimations. A potentially increasing relative contribution from VLDL FA with increased time post-feeding may also play a role. Thus whether predictions would be similarly improved in the largely post-absorptive lipoproteins at 12 h is not known.

It is clear from this work that in order to use chylomicron FA signatures to estimate diet, chylomicrons must be visibly present and isolated from the rest of the blood sample prior to FA analysis. If whole blood, plasma or serum were analyzed the increased input of endogenous FA, carried in the other lipoprotein classes, would lead to highly erroneous results in the modeling of diets. Provided moderate levels of visible chylomicrons are present, one can be relatively confident in obtaining useful model predictions of diet, even in free-ranging animals when time since feeding is unknown. I also suggest that since endogenous FA associated with chylomicron PL introduce noise in diet estimates even when chylomicrons are largest and most abundant, isolating the FA in TAG from those in PL should further improve results and provide the most reliable estimates of diets from chylomicron FA.

Chapter 4. Body Composition and Energy Balance in Captive, Juvenile Grey Seals Intubated with Experimental Diets

Introduction

In pinnipeds, controlled feeding studies have been used to assess metabolic rates, digestive efficiencies and maintenance requirements and are crucial to the development and validation of QFASA (Keiver *et al.* 1984, Ronald *et al.* 1984, Lawson *et al.* 1997a,b, Rosen and Trites 2000a, Kirsch *et al.* 2000, Tollit *et al.* 2003, Iverson *et al.* 2004). Such studies require large aquarium-type facilities and often animals housed therein are primarily in captivity for public exhibition and/or education purposes, making it difficult to fully control studies and to repeatedly sample individuals. Additionally, in such "controlled diet" studies, seals are generally fed individual whole fish from a given lot or lots and then separate fish from those lots are used for analyses of proximate and FA composition (Kirsch *et al.* 2000, Tollit *et al.* 2003, Iverson *et al.* 2004). However, individual fish of a species, even within lots, can vary substantially in fat content and FA composition (Jangaard 1974, Montevecchi and Piatt 1984, Stansby 1986, Mårtensson *et al.* 1996, Budge *et al.* 2002, Iverson *et al.* 2002), such that the exact dietary intake is not known. Given these constraints, I designed a means by which free-ranging grey seals could be brought into "captivity" for short-duration feeding experiments in which completely homogenous diets could be fed to individuals. In these studies, homogenous fish-based diets were intubated to juvenile grey seals. The primary purpose of these studies was to further develop the QFASA method using precise knowledge of the FA composition consumed by the seals. However, because this method of feeding had not

been evaluated, an important goal of this research was to determine how animals would respond to such feeding regimes and diets and to assess whether it could be a viable option for future captive feeding studies. Thus, I also used these studies to assess energy balance and body composition changes and to examine the way in which the daily energy budget is partitioned into growth, maintenance and fattening.

Juvenile pinnipeds are an important age class to study in terms of their energy requirements. Because they are rapidly growing animals, they have high energy requirements for their body size (Keiver *et al.* 1984, Ronald *et al.* 1984, Boyd 2002, Winship *et al.* 2002). This, in addition to their limited dive capabilities (Thorson and LeBoeuf 1994, Burns and Castellini 1996, Horning and Trillmich 1997), should make juveniles particularly susceptible to changes in prey availability and abundance (Trillmich and Limberger 1985, Merrick and Loughlin 1997, Horning and Trillmich 1999). It is important to understand the conditions affecting juvenile survival because juvenile recruitment is an important factor affecting pinniped population dynamics (Eberhardt and Siniff 1977, York 1994, NRC 1996). Their high relative energy requirements and abundance within populations also means that the prey consumed by juveniles can represent the largest portion of food consumption by a given pinniped population (Mohn and Bowen 1996, Winship *et al.* 2002), giving this age class a particularly strong impact on prey populations.

The partitioning of daily energy budgets was studied by monitoring changes in mass and body composition throughout controlled feeding experiments. Body composition is typically estimated *in vivo* from measurements of total body water (TBW) using hydrogen isotope (deuterium or tritium) dilution methods. A major assumption in

this methodology is that the tracer only mixes with TBW. It has been known for some time, however, that some hydrogen isotope is lost to rapidly exchangeable hydrogen atoms in organic constituents of the body (Ussing 1935, Culebras and Moore 1977, Culebras *et al.* 1977, Schoeller *et al.* 1980). Because the loss of this hydrogen isotope lowers the equilibrium isotope concentration of the aqueous phase, hydrogen-isotope dilution space will tend to overestimate TBW. It is, therefore, necessary to apply a correction factor to dilution space in order to arrive at a measure of TBW.

Culebras and Moore (1977) found that the theoretical maximum overestimation of TBW measurements by isotope dilution was 5.22%, but empirical studies show that this maximum is rarely reached (Culebras *et al.* 1977, Nagy and Costa 1980, Reilly and Fedak 1990, Lydersen *et al.* 1992, Oftedal *et al.* 1993, Farley and Robbins 1994, Arnould *et al.* 1996, Oftedal *et al.* 1996). Regression equations relating measurements of hydrogen-isotope dilution space to TBW are derived from validation studies in which measurements of TBW by isotope dilution are followed by carcass desiccation of the same individual. The large size of pinnipeds makes validation studies difficult to carry out, and it is unlikely that regression equations can be developed for all species. However, Bowen and Iverson (1998) were able to fit all existing data on pinnipeds to a single predictive equation that can be used to estimate TBW in species that lack empirical data.

Once an estimate of TBW is made, regression equations based on this value and a measure of body mass taken at the time of isotope dilution can be used to predict total body fat (TBF) and total body protein (TBP), given the relative constancy of water and protein contents of lean body mass in mammals of a given species and age. Reilly and

Fedak (1990) recommend using a regression equation that has an empirical basis in the species under investigation, as interspecific extrapolation of these equations reduces the accuracy of predictions (Reid *et al.* 1955, Viljoen *et al.* 1988). Predictive equations, based on chemical analysis of carcasses, have been derived for harp seals (Gales *et al.* 1994), ringed seals *(Phoca hispida*: Stirling and McEwan 1975), grey seals (Reilly and Fedak 1990), and Antarctic fur seals (*Arctocephalus gazella*: Arnould *et al.* 1996).

Hydrogen-isotope dilution methods have also been used widely to estimate milk or solid-food intake and energy metabolism in pinnipeds (Costa 1987, Oftedal and Iverson 1987). To measure food intake (FI), total water intake (TWI) must be determined. TWI includes both preformed water in the diet and metabolic water produced from the catabolism of diet constituents and/or body nutrient stores (Oftedal and Iverson 1987). FI is then calculated from an equation relating TWI to FI that is based on measures of daily fat and protein deposition as well as the percent water, protein and fat content of the diet.

Methods

Animal Maintenance and Sampling

Juvenile grey seals (5-10 months) were captured from the beaches of Sable Island, Nova Scotia, Canada in spring 1998 (n = 4), spring 1999 (n = 8 and n = 8), fall 1999 (n = 8) and spring 2000 (n = 10 and n = 10). These seals were used in 6 independent experiments. Seals were placed in large pens (approximately 5 x 4 m, 2-3 seals to each pen) located high on the beach. Each pen was covered fully with a vinyl tarp with rivet

holes throughout, blocking direct sunlight but allowing rain to drain through. A large fire hose, reaching from the ocean to the pens, was attached to a water pump, with the intake end anchored in the shallow water off the beach. This pump and hose system was used to deliver seawater to fill storage reservoirs nearby the pens and to fully immerse seals twice daily in shallow water pools.

Before the beginning of each diet trial the seals were fasted overnight. On the first day (0 d), the mass of each seal was measured to the nearest 0.5 kg on a 100-kg Salter scale. Total body water and water turnover rate were measured by isotope dilution. A precisely weighed quantity (3g/kg body mass) of deuterium oxide (D_2O, 99.8 atom%, Sigma Aldrich) was administered to each seal from a 60-cm^3 syringe with a 16 French gastric tube. The syringe and gastric tube were rinsed (via 3-way stopcocks) with two 5-cm^3 aliquots of distilled water, and air was blown through the tube as it was withdrawn to ensure complete isotope delivery. Seals did not have access to food or water for at least 3 h to allow the isotope to equilibrate. To determine the equilibrium concentration of D_2O, serial blood samples (~ 10-cm^3) were taken from the extradural vein approximately 0.5 h apart and at least 2.5 h after isotope administration.

At the approximate midpoint of each experiment, seals were re-weighed and a blood sample was taken to measure residual isotope level. In the Spring 2000 experiments, mass and blood samples were also taken on 3 d to allow for comparison between body composition and water turnover parameters from periods of partial and full food rations. On the last day of each experiment, an initial blood sample was taken to measure residual isotope concentration and another dose of D_2O (1g/kg body mass) was

administered and allowed to equilibrate. Equilibrium blood samples were then taken as above.

Homogenous diets were prepared daily from ground fish (Spring 1998, Fall 1999, Spring 2000) or pelleted fish-meal (Spring 1999), and supplemented with fish oil. Diets were sub-sampled each day for compositional analysis. Seals were restrained using a net and fed by gastric intubation using a ¾ inch foal stomach tube. Each seal was assigned a food container, the contents of which were weighed before and after each feeding, the mass difference constituting that meal. These mass estimates were used to compare with the food consumption rates measured by isotope dilution. On 12 d of the Spring 2000A experiment, faecal samples were collected from 6 of the 10 animals and percent lipid content determined, providing an indication of how well the animals were able to absorb the lipid from the diet.

The amount of food to be fed to the seals each day was determined based on expected maintenance energy requirements. The equation used to calculate the gross energy requirement is:

$$GEI = FE + UE + HIF + (BMR + T + L)$$

where GEI is gross energy intake, FE is faecal energy loss, UE is urinary energy loss, HIF is the heat increment of feeding, BMR is the basal metabolic rate, T is energy required for thermoregulation and L is cost of locomotion.

Gallivan and Ronald (1981) found that the HIF of harp seals on a herring diet accounted for 17% of ingested energy. Rosen and Trites (2000b) found a similarly high value of HIF in Steller sea lions consuming a pollock only diet. These values were much higher than those found for harbour seals on either a herring or a pollock diet (Ashwell-

Erikson and Elsner 1981) or Steller sea lions consuming a herring diet (Rosen and Trites 1997). To be conservative, I used the highest value in the calculation of gross energy requirement. The digestible energy is gross energy minus the amount of energy lost to faeces, whereas, metabolizable energy (ME) gives an indication of the total energy lost to both faeces and urine. I assumed ME to be approximately 85% of gross energy intake, consistent with values found for pinnipeds consuming various prey (Keiver *et al.* 1984, Ronald *et al.* 1984, Mårtensson *et al.* 1994, Lawson *et al.* 1997a,b, Rosen and Trites 2000a). The most widely used equation relating basal metabolic rate and body size, for mammals, is that given in Kleiber (1975): $BMR = 3.4M^{0.75}$ where BMR is in watts and M is body mass in kilograms. In general, immature mammals have metabolic requirements approximately two times that predicted for adult mammals of the same size (Brody 1945). This is a result of the increased amounts of energy needed for growth in the juvenile relative to the adult. This doubling of the metabolic rate in juveniles has been found to be true for marine mammals (Ronald *et al.* 1984, Lavigne *et al.* 1986, Worthy 1987). The thermoneutral zone of juvenile grey seals, in air, is -18° to 30° C (Boily and Lavigne 1996). The temperatures experienced on Sable Island in spring and fall are well within this range and my study animals were relatively inactive so both T and L were considered equal to zero.

Maintenance is defined as the amount of ingested energy (IE) necessary for an animal neither to gain nor lose body weight (Kleiber 1975). The calculation of the amount of ingested energy required for the maintenance of a 40 kg juvenile grey seal, therefore, becomes: $IE = (0.15xIE) + (0.17xIE) + 2[3.4(40)^{0.75}]$. This is equivalent to approximately 13.7 MJ/d. Based on this estimate of the MR and the expected energy

content of the diets (~ 9.5 MJ/kg if diet is 15% fat and 15% protein), maintenance food requirements were approximately 1.5 kg/d. Because I wanted the study animals to gain mass and fat, I fed 1.5x the maintenance energy requirement, which corresponded to approximately 2.2 kg/d.

No effect of meal size on the efficiency with which food is digested was found in ringed seals (Lawson *et al.* 1997a), harp seals (Lawson *et al.* 1997b), or Steller sea lions (Rosen *et al.* 2000) when meal sizes ranging between 0.75% and 4.5% body mass were tested. Adjustments in digestive capacity, in response to increased food intake, may explain these findings (Cripps and Williams 1975, Barry 1976, Gross *et al.* 1985, Lloyd *et al.* 1994). Although Warner (1981) concluded that food given in frequent meals moves more rapidly through the digestive tract than that given infrequently at the same ration, and meal-fed rats (Leveille and Hanson 1966) and pigs (Allee *et al.* 1972) are more efficient in food utilization than animals fed *ad libitum*, feeding frequency does not appear to have an effect on digestible or metabolizable energy in pinnipeds (Keiver *et al.* 1984, Rosen *et al.* 2000). Keiver *et al.* (1984), however, did show that maintenance energy requirements are increased when four meals are fed per day compared to two and they suggested this was a result of increased activity levels associated with the increased feeding frequency. In light of this and considering the greater amount of stress that would likely be experienced by seals during intubation of a meal, I chose a feeding regime of only two meals per day. Sufficient volume to achieve the required daily energy intakes could not be handled by seals in a single feeding by gastric intubation. For the first three days of each experiment (1-3 d) seals were fed partial rations to allow them to adjust to their new diet. From 3 d to the end of each experiment, seals were fed twice

daily for total intakes of approximately 2.2 kg/d. Seals were also given vitamin (B_1, B/C complex, and vitamin E) and salt supplements.

Laboratory Procedures

Blood samples were immediately taken to the field laboratory where they were refrigerated (4°C) for a maximum of 8 hours. Samples were then centrifuged at 2000 rpm for 20 min. Serum was removed and stored frozen (-20°C) in 5 ml cryovials until analysis (< 6 months later). Total free water was then collected from serum samples by heat distillation (Oftedal and Iverson 1987). The concentration of D_2O was determined by quantitative infrared spectrophotometry on a Perkin-Elmer 283B infrared spectrophotometer as described by Oftedal and Iverson (1987).

Homogenous diet samples, collected each day of feeding, were analyzed in duplicate for dry matter (by oven drying at 105°C for 5 hours), ash (by dry ashing in a Muffle Furnace at 550°C), protein (by micro Kjeldahl method), and fat (using a modified Folch method outlined in Iverson *et al.* 2001).

Isotope Calculations

Isotope equilibration was considered to have occurred if the isotope levels of the two sequential blood samples were within 0.01% of each other. According to this criterion, all animals, on each administration day, were considered to have equilibrated by 2.5 h. Isotope dilution space (D), on the initial and final sampling days, was calculated using the equilibrated isotope concentrations (C), as described by Iverson *et al.* (1993):

$$D_{initial} = \frac{gD_2O \text{ administered}}{10 \times C_{initial}} \qquad D_{Final} = \frac{gD_2O \text{ re-administered}}{10 \times (C_{final} - C_{initial})}$$

Dilution space was converted to total body water (TBW, kg) using the equation developed by Bowen and Iverson (1998) which gives an approximately 3% correction: TBW = 0.003 + 0.968(D). Estimates of TBW on intermediate days were made with the assumption that the change in TBW was proportional to the change in mass (M) (Oftedal and Iverson 1987). The following formula was used:

$$TBW_t = TBW_{initial} - [(M_{initial} - M_t) \times [(TBW_{initial} - TBW_{final})/(M_{initial} - M_{final})]]$$

In the Spring 2000 data, the TBW interpolations appeared spurious for the 3 d values. Between the initial and final sampling days in this experiment, TBW changed substantially with only a small change in mass; thus, the interpolation equation gave very large TBW changes with the mass decreases between 0-3 d. When converted to TBF measurements, this suggested that seals lost mass but gained fat, which is not possible. An alternative calculation was derived from data on juvenile harbour seals, which had begun to feed but were not yet able to maintain mass (Muelbert *et al.* 2003). Those animals exhibited body composition changes similar to those seen in my study animals during the first three days of the Spring 2000 feeding trials; they experienced mass loss but %TBW gains. A Δ%TBW coefficient was calculated from the harbour seal data as follows:

$$\Delta\ \%TBW = (\%TBW_{final} - \%TBW_{initial})/days$$

This coefficient was applied to the Spring 2000 grey seal data to calculate the 3 d %TBW values as follows:

$$\%TBW_{Day\ 3} = \%TBW_{initial} + (\Delta\ \%TBW \times days)$$

Body composition was calculated from mass and %TBW, using regression equations derived for grey seals by Reilly and Fedak (1990). TBP and TBF were calculated as a percentage of body mass using the following equations:

$$\%TBP = 0.42(\%TBW) - 4.75$$

$$\%TBF = 105.1 - 1.465(\%TBW)$$

TWI (kg/d) and FI (kg/d) were calculated for each animal from isotope dilution data for comparison with delivery estimates. First, serum isotope concentrations were corrected for changes in TBW from 0 d according to Iverson *et al.* (1993). This was necessary because TBW was not constant in these animals and an increase in TBW causes a decline in isotope concentration that is not due to turnover (Nagy and Costa 1980, Oftedal and Iverson 1987). Fractional turnover rate (k) of isotope was then calculated from the linear regression of the natural logarithm of the corrected isotope concentrations against time elapsed since D_2O administration. TWI was determined as the sum of the total water loss (k x $TBW_{average}$) and total water gain or loss over the time period (ΔTBW) as described in Oftedal *et al.* (1987). TWI was then converted to FI using the equation:

$$FI = \frac{100 \times [TWI + 1.07(F_{Dep.}) + 0.42(P_{Dep.})]}{[\%W_{Diet} + 0.995(\%F_{Diet}) + 0.391(\%P_{Diet})]}$$

where F_{Dep} and P_{Dep} represent daily fat and protein deposition (kg/d), respectively, of individual seals, and $\%W_{Diet}$, $\%F_{Diet}$, and $\%P_{Diet}$ represent water, fat and protein content of the diet, respectively. This equation was derived from Oftedal *et al.* (1987) and modified assuming 93% digestible energy of the diet as found by Ronald *et al.* (1984) for juvenile grey seals eating a herring diet. FI was converted to GEI (MJ/d) using the factors of 39.3 MJ/kg fat and 23.6 MJ/kg protein (Schmidt-Nelson 1979).

Data Analysis

Mass changes over the course of each experiment were estimated by repeated-measures ANOVA. Differences in TBW between initial and final measurements were tested by two-tailed paired t-tests. Comparisons between the field estimates of food intake and those calculated from isotope dilution were made using two-tailed paired t-tests. Water turnover rate as well as the mass, fat and protein deposition rates in individual periods of each experiment were tested for differences using one-tailed paired t-tests except in the case of the Spring 2000 experiments which, because there were three periods, were tested using repeated-measures ANOVA.

Maintenance energy requirements were calculated from the regression of gross energy intake per metabolic size (MJ * $kg^{-0.75}$ * d^{-1}) against mass change (kg * d^{-1}). Maintenance energy per metabolic size declines with age (Kleiber 1975) so values were calculated for the 5-month old seals and the 10-month old seals separately.

Results

Diet Composition

Because this study represented a new approach to controlled feeding studies, experimental diets were necessarily developed through a combination of trial and error and availability of feed sources. In Spring 1998, the feeding method itself was tested in a small trial using fish-based meals and large supplements of oil. This proved to be extremely cumbersome in terms of transporting huge amounts of frozen fish to Sable Island and in daily preparation. The amount of oil was also deemed too high, given

animal responses (see below), thus implying even greater transport of frozen fish required. Thus, I sought out a new source for the first full-trial experiments (Spring 1999). Pelleted fish meal was purchased from Moore-Clark, Inc. and seemed an ideal solution, as it could easily be mixed into a homogenous slurry supplemented with limited oil. Unfortunately, animals responded very poorly, the cause of which we now know (see below). Thus, beginning in the fall of 1999, I returned to a whole fish-based diet (Table 4.1).

As a consequence of the changing formulations required, the proximate composition (on a wet-weight basis) and energy contents of experimental diets varied considerably (Table 4.1). The diet used in Spring 1998 had the highest fat content (24.5%) and, correspondingly, the highest energy (11.1 MJ/kg) but lowest protein (8.3%) content. The two Spring 1999 diets had the highest unidentified material at 8-11%. The Fall 1999 diet had the lowest fat (9.7%) and energy (6.4 MJ/kg) contents. The diets used in the two Spring 2000 experiments were quite similar in composition and energy content, and intermediate relative to the diets of Spring 1998 and Fall 1999. Dry matter varied across the diets with the highest value associated with the Spring 1998 diet (39.7%) and the lowest associated with the Fall 1999 diet (26.6%).

As stated above, both diets used in Spring 1999 contained pelleted fish meal, as opposed to ground fresh-frozen fish, as their protein source. Consumption of these diets led to severe adverse health effects and, in several cases, death. After necropsy and communication with Dr. Chris Harvey-Clark (Dalhousie University Veterinarian) clostridial enteritis was suspected. The high level of unidentified material in these diets was likely responsible for this development of clostridial enteritis in several, if not all, of

Table 4.1: Diet components, proximate composition (wet weight) and energy content of experimental diets (mean ± SE).

Experiment	Protein Source	Fat Supplement	Fat	Protein	Dry Matter	Ash	Unidentified	Energy
			%	%	%	%	%	(MJ/kg)
Spring 1998	Ground Herring	Menhaden Oil	24.5 ± 0.69	8.3 ± 0.61	39.7 ± 0.50	2.4 ± 0.10	4.5 ± 0.61	11.1 ± 0.19
Spring 1999A	Pelleted Fish-Meal	Menhaden Oil	12.0 ± 0.62	12.7 ± 0.83	34.2 ± 1.88	1.3 ± 0.08	8.2 ± 0.97	7.7 ± 0.29
Spring 1999B	Pelleted Fish-Meal	Menhaden Oil	10.5 ± 0.55	13.8 ± 0.33	36.4 ± 0.43	1.5 ± 0.03	10.7 ± 0.39	7.4 ± 0.19
Fall 1999	Ground Cod	Anchovy Oil	9.7 ± 0.47	11.1 ± 0.13	26.6 ± 0.58	3.5 ± 0.15	2.4 ± 0.42	6.4 ± 0.18
Spring 2000A	Ground Cod	Capelin Oil	14.9 ± 0.71	9.4 ± 0.21	32.6 ± 0.70	3.7 ± 0.18	4.6 ± 0.64	8.1 ± 0.26
Spring 2000B	Ground Cod	Herring Oil	13.2 ± 0.62	9.7 ± 0.16	30.1 ± 0.64	4.5 ± 0.18	2.6 ± 0.58	7.5 ± 0.24

the seals. Of the 8 seals in each of the Spring 1999 experiments, only 3 and 4 completed the first and second experiments, respectively.

Estimates of Food Intake

The field estimates of food intake were generally similar to those calculated from isotope dilution, however, in several cases significant differences were found. Field estimates were significantly lower in the second period of Spring 1998 (1.33 ± 0.076 kg/d vs. 1.96 ± 0.100 kg/d, $p = 0.025$) and Spring 1999B (1.83 ± 0.077 kg/d vs. 2.45 ± 0.113 kg/d, $p = 0.001$), and the first period of Spring 1999A (1.99 ± 0.118 kg/d vs. 2.26 ± 0.203 kg/d, $p = 0.044$) and Fall 1999 (1.95 ± 0.016 kg/d vs. 2.28 ± 0.058 kg/d, $p = 0.002$). Field estimates of food intake were significantly higher in the second and third periods of Spring 2000A (3.70 ± 0.038 kg/d vs. 3.31 ± 0.097 kg/d, $p = 0.001$ and 3.94 ± 0.067 vs. 3.20 ± 0.086 kg/d, $p < 0.001$, respectively). Field estimates of food delivery were hindered by food spillage, adherence to the stomach tube and by occasional regurgitation. Given their greater reliability, I used only measures of food intake from isotope dilution in further analyses.

Dietary Intakes and Body Compositional Changes

Spring 1998

Seals tended to lose mass over the course of the Spring 1998 trial experiment with the greatest loss occurring in the first period, however, these losses were not significant (Table 4.2). The loss of TBW between the initial and final sampling periods was both significant and greater than the mass loss. This decrease in %TBW resulted in an

increase in the value of %TBF and a decrease in the value of %TBP, which translated into an increase of 1.9 kg in TBF and a decrease of 1.2 kg in TBP. Within the first sampling period, however, the opposite pattern of change was seen. The loss of TBW was smaller than the mass loss such that the %TBW increased, producing absolute and proportional fat losses and proportional protein gains. The water turnover rate was significantly smaller in the first period of the experiment compared to the second (Table 4.3). This corresponded to a doubling of food, nutrient and energy intakes during the second time period relative to the first. The rate of mass loss was smaller in the second period of the experiment, in accordance with the higher rates of intake. The fat deposition rate was significantly different, changing from a negative value in the first period to a positive value in the second period. The rate of protein deposition was similar in the two periods of measurement.

Spring 1999

There were no significant changes in mass and body composition parameters of the few seals that completed either of the Spring 1999 experiments (Tables 4.4 and 4.6). In Spring 1999A there were slight increases in mass and TBW, the increases being larger in the second period of the experiment (Table 4.4). Body composition parameters (%TBW, %TBF, %TBP) were virtually unchanged, leading to small absolute fat and protein gains. The water turnover rate was significantly greater in the second period of the experiment relative to the first, producing higher values for food, nutrient and energy intake rates (Table 4.5). Mass, fat and protein deposition rates, however, did not differ significantly between the two periods of measurement. In the initial period of Spring 1999B mass declined but between the beginning and end of the experiment, mass

Table 4.2: Mass and body composition of grey seals (n = 3) during Spring 1998. Values are means ± SE. Mass differences tested by repeated-measures ANOVA. Differences in TBW between initial and final measurements tested by a two-tailed paired t-test.

	Mean	±	SE	*P*	F
Mass (kg):					
0d	34.0	±	1.89		
5d	32.2	±	2.13		
13d	31.8	±	2.46	0.078	11.39
TBW (kg):					
0d	19.7	±	0.55		
5d	19.2	±	0.78		
13d	16.8	±	0.81	0.01*	
TBW (%):					
0d	58.2	±	1.86		
5d	60.0	±	1.71		
13d	53.1	±	2.34		
TBF (kg):					
0d	6.9	±	1.20		
5d	5.6	±	1.16		
13d	8.8	±	1.63		
TBF (%):					
0d	20.0	±	2.54		
5d	17.2	±	2.51		
13d	27.3	±	3.43		
TBP (kg):					
0d	6.7	±	0.15		
5d	6.5	±	0.22		
13d	5.5	±	0.24		
TBP (%):					
0d	19.7	±	0.74		
5d	20.5	±	0.74		
13d	17.5	±	0.96		

* Significant at the 0.05 level after Bonferroni adjustment for multiple comparisons.

Table 4.3: Water, food, and energy intake and nutrient deposition during the first and second parts of the experimental period of Spring 1998 (n = 3). Values are means ± SE.

	0-5 d		5-13 d		*P*
Water turnover rate (*k*)	0.062 ±	0.0060	0.090 ±	0.0017	0.012*
Intake:					
Total water intake (kg/d)	1.11 ±	0.043	1.30 ±	0.081	
Food intake (kg/d)	0.94 ±	0.074	1.96 ±	0.100	
Fat intake (kg/d)	0.23 ±	0.019	0.48 ±	0.025	
Protein intake (kg/d)	0.08 ±	0.003	0.16 ±	0.010	
Energy intake (MJ/d)	10.38 ±	0.801	21.75 ±	1.123	
Energy intake (MJ/kg0.75/d)	0.77 ±	0.087	1.60 ±	0.021	
Deposition:					
Δ Mass (kg/d)	-0.38 ±	0.088	-0.04 ±	0.042	0.017
Fat deposition (kg/d)	-0.26 ±	0.022	0.41 ±	0.101	0.010*
Protein deposition (kg/d)	-0.02 ±	0.023	-0.04 ±	0.009	0.257

* Significant at the 0.05 level after Bonferroni adjustment for multiple comparisons.

increased slightly (Table 4.6). TBW decreased leading to a decrease in %TBW and %TBP, and an increase in %TBF. These proportional changes corresponded to similar absolute changes in TBF and TBP. Once again, the water turnover rate was greater in the second period of Spring 1999B relative to the first but in this case the difference was not significant (Table 4.7). The rate of mass change differed significantly between the two periods of the experiment with a negative value in the first period and a positive value in the second. The rates of fat and protein deposition, however, were not significantly different between the two periods of the experiment.

Fall 1999

Over the course of the Fall 1999 experiment significant gains were recorded for both mass and TBW, all or most of which were made in the second period of the experiment (Table 4.8). This led to an increase in %TBW, which translated into absolute and proportional fat losses and protein gains. The water turnover rate was significantly greater in the second period of this experiment compared to the first (Table 4.9). This led to an increase in the rates of food, nutrient and energy intakes. Deposition rates were also greater in the second period of the experiment, significantly so in the case of mass and protein changes.

Table 4.4: Mass and body composition of grey seals (n = 3) during Spring 1999A. Values are means ± SE. Mass differences tested by repeated-measures ANOVA. Differences in TBW between initial and final measurements tested by a two-tailed paired t-test.

	Mean	±	SE	*P*	F
Mass (kg):					
0d	38.3	±	1.09		
6d	39.5	±	1.61		
14d	41.7	±	1.17	0.008	20.60
TBW (kg):					
0d	21.3	±	1.39		
6d	21.9	±	1.59		
14d	23.0	±	1.29	0.071	
TBW (%):					
0d	55.4	±	2.21		
6d	55.3	±	2.13		
14d	55.3	±	2.47		
TBF (kg):					
0d	9.1	±	1.04		
6d	9.4	±	0.96		
14d	10.0	±	1.44		
TBF (%):					
0d	24.0	±	3.21		
6d	24.0	±	3.10		
14d	24.1	±	3.61		
TBP (kg):					
0d	7.1	±	0.52		
6d	7.3	±	0.59		
14d	7.7	±	0.50		
TBP (%):					
0d	18.5	±	0.93		
6d	18.5	±	0.89		
14d	18.4	±	1.03		

* Significant at the 0.05 level after Bonferroni adjustment for multiple comparisons.

Table 4.5: Water, food and energy intake and nutrient deposition during the first and second parts of the experimental period of Spring 1999A (n = 3). Values are means ± SE.

	0-6 d		6-14 d		*P*
Water turnover rate (*k*)	0.079 ±	0.0021	0.094 ±	0.0031	0.002*
Intake:					
Total water intake (kg/d)	1.81 ±	0.123	2.17 ±	0.057	
Food intake (kg/d)	2.26 ±	0.203	2.75 ±	0.052	
Fat intake (kg/d)	0.27 ±	0.023	0.33 ±	0.006	
Protein intake (kg/d)	0.29 ±	0.026	0.35 ±	0.006	
Energy intake (MJ/d)	17.43 ±	1.574	21.24 ±	0.387	
Energy intake (MJ/kg0.75/d)	1.13 ±	0.081	1.35 ±	0.030	
Deposition:					
Δ Mass (kg/d)	0.19 ±	0.099	0.27 ±	0.083	0.350
Fat deposition (kg/d)	0.04 ±	0.081	0.08 ±	0.067	0.330
Protein deposition (kg/d)	0.04 ±	0.023	0.05 ±	0.013	0.360

* Significant at the 0.05 level after Bonferroni adjustment for multiple comparisons.

Table 4.6: Mass and body composition of grey seals (n = 4) during Spring 1999B. Values are means ± SE. Mass differences tested by repeated-measures ANOVA. Differences in TBW between initial and final measurements tested by a two-tailed paired t-test.

	Mean	±	SE	*P*	F
Mass (kg):					
0d	37.4	±	0.66		
8d	36.4	±	0.55		
19d	37.6	±	0.38	0.090	3.71
TBW (kg):					
0d	20.8	±	0.52		
8d	20.0	±	0.81		
19d	19.9	±	0.53	0.322	
TBW (%):					
0d	55.6	±	1.67		
8d	55.2	±	2.93		
19d	52.8	±	1.63		
TBF (kg):					
0d	8.9	±	0.96		
8d	8.9	±	1.64		
19d	10.5	±	1.01		
TBF (%):					
0d	23.7	±	2.44		
8d	24.3	±	4.28		
19d	27.8	±	2.49		
TBP (kg):					
0d	7.0	±	0.22		
8d	6.7	±	0.35		
19d	6.6	±	0.24		
TBP (%):					
0d	18.6	±	0.71		
8d	18.5	±	1.22		
19d	17.4	±	0.70		

* Significant at the 0.05 level after Bonferroni adjustment for multiple comparisons.

Table 4.7: Water, food and energy intake and nutrient deposition in the first and second parts of the experimental period of Spring 1999B (n = 4). Values are means ± SE.

	0-8 d	8-19 d	*P*
Water turnover rate (*k*)	0.076 ± 0.0060	0.098 ± 0.0027	0.016
Intake:			
Total water intake (kg/d)	1.45 ± 0.039	1.94 ± 0.092	
Food intake (kg/d)	1.82 ± 0.135	2.45 ± 0.113	
Fat intake (kg/d)	0.19 ± 0.014	0.26 ± 0.012	
Protein intake (kg/d)	0.25 ± 0.018	0.34 ± 0.017	
Energy intake (MJ/d)	13.43 ± 0.997	18.08 ± 0.838	
Energy intake (MJ/kg0.75/d)	0.89 ± 0.053	1.23 ± 0.071	
Deposition:			
Δ Mass (kg/d)	-0.13 ± 0.031	0.11 ± 0.050	0.001*
Fat deposition (kg/d)	0.00 ± 0.105	0.15 ± 0.104	0.215
Protein deposition (kg/d)	-0.03 ± 0.032	-0.01 ± 0.028	0.328

* Significant at the 0.05 level after Bonferroni adjustment for multiple comparisons.

Table 4.8: Mass and body composition of grey seals (n = 8) during Fall 1999. Values are means ± SE. Mass differences tested by repeated-measures ANOVA. Differences in TBW between initial and final measurements tested by a two-tailed paired t-test.

	Mean	±	SE	*P*	F
Mass (kg):					
0 d	38.6[a]	±	1.04		
9 d	38.6[a]	±	0.91		
15 d	40.1[b]	±	0.97	0.001*	12.87
TBW (kg):					
0 d	23.5	±	0.60		
9 d	23.7	±	0.59		
15 d	24.8	±	0.70	0.006*	
TBW (%):					
0 d	61.0	±	0.48		
9 d	61.4	±	0.47		
15 d	61.8	±	0.77		
TBF (kg):					
0 d	6.1	±	0.36		
9 d	5.9	±	0.30		
15 d	5.8	±	0.47		
TBF (%):					
0 d	15.8	±	0.71		
9 d	15.2	±	0.69		
15 d	14.5	±	1.13		
TBP (kg):					
0 d	8.0	±	0.21		
9 d	8.1	±	0.21		
15 d	8.5	±	0.25		
TBP (%):					
0 d	20.9	±	0.20		
9 d	21.0	±	0.20		
15 d	21.2	±	0.33		

* Significant at the 0.05 level after Bonferroni adjustment for multiple comparisons.
[ab] Values with different superscript letters differ significantly.

Table 4.9: Water, food and energy intake and nutrient deposition during the first and second parts of the experimental period of Fall 1999 (n = 8). Values are means ± SE.

	0-9 d		9-15 d		*P*
Water turnover rate (*k*)	0.085 ±	0.0010	0.104 ±	0.0061	0.010*
Intake:					
Total water intake (kg/d)	2.01 ±	0.064	2.66 ±	0.087	
Food intake (kg/d)	2.28 ±	0.058	3.07 ±	0.136	
Fat intake (kg/d)	0.22 ±	0.006	0.30 ±	0.013	
Protein intake (kg/d)	0.25 ±	0.006	0.34 ±	0.015	
Energy intake (MJ/d)	14.52 ±	0.370	19.57 ±	0.863	
Energy intake (MJ/kg0.75/d)	0.95 ±	0.014	1.28 ±	0.075	
Deposition:					
Δ Mass (kg/d)	-0.01 ±	0.038	0.27 ±	0.041	0.001*
Fat deposition (kg/d)	-0.04 ±	0.022	0.02 ±	0.065	0.489
Protein deposition (kg/d)	0.00 ±	0.009	0.07 ±	0.011	0.001*

* Significant at the 0.05 level after Bonferroni adjustment for multiple comparisons.

Spring 2000

Between the beginning and end of Spring 2000A, significant gains were recorded for both mass and TBW, however, both parameters dropped during the first period of the experiment (Table 4.10). The overall gains in mass and TBW led to an increase in %TBW, which translated into absolute and proportional fat losses and protein gains. Mass and TBW showed identical patterns of change in Spring 2000B, however, in this case none of the changes were significant (Table 4.12). The water turnover rate during the initial food acclimation period (0–3 d) was significantly lower than that in the two periods of full ration feeding for both Spring 2000A and Spring 2000B (Tables 4.11 and 4.13). This led to a dramatic increase in the calculated food, nutrient and energy intakes starting in the second period of each experiment. The intake rates were very similar between the second and third periods of Spring 2000A (Table 4.11) whereas a slight drop in intake rates was apparent in the third period of Spring 2000B (Table 4.13). In Spring 2000A, the mass, fat and protein deposition rates were significantly lower during the first period of the experiment (Table 4.11). The mass and protein deposition rates did not differ between the two later periods but the fat deposition rate was significantly lower in the third period relative to the second. In Spring 2000B, the rate of mass change was similar in the first and third periods but significantly greater during the second period (Table 4.13). The fat and protein deposition rates did not differ between any of the periods of the experiment. The faecal lipid content, of samples collected on 12 d of the Spring 2000A experiment, ranged from 0.81-14.6% with an average of 4.7 ± 1.67 %.

Table 4.10: Mass and body composition of grey seals (n = 10) during Spring 2000A. Values are means ± SE. Mass differences tested by repeated-measures ANOVA. Differences in TBW between initial and final measurements tested by a two-tailed paired t-test.

	Mean	±	SE	*P*	F
Mass (kg):					
0d	37.9[a]	±	2.08		
3d	36.35[b]	±	2.11		
11d	38.25[ac]	±	2.14		
17d	39.1[c]	±	2.05	0.005*	8.52
TBW (kg):					
0d	21.2	±	1.19		
3d	20.7	±	1.22		
11d	22.0	±	1.28		
17d	23.0	±	1.30	<0.001*	
TBW (%):					
0d	55.9	±	0.74		
3d	57.0	±	0.74		
11d	57.4	±	0.57		
17d	58.8	±	0.65		
TBF (kg):					
0d	8.8	±	0.62		
3d	7.9	±	0.59		
11d	8.0	±	0.50		
17d	7.3	±	0.46		
TBF (%):					
0d	23.3	±	1.08		
3d	21.7	±	1.07		
11d	21.1	±	0.84		
17d	18.9	±	0.95		
TBP (kg):					
0d	7.1	±	0.40		
3d	7.0	±	0.41		
11d	7.4	±	0.44		
17d	7.8	±	0.45		
TBP (%):					
0d	18.7	±	0.31		
3d	19.2	±	0.31		
11d	19.3	±	0.24		
17d	20.0	±	0.27		

* Significant at the 0.05 level after Bonferroni adjustment for multiple comparisons.
[abc] Values with different superscript letters differ significantly.

Table 4.11: Water, food and energy intake and nutrient deposition during the first, second and third parts of the experimental period of Spring 2000A (n = 10). Values are means ± SE.

	0-3d	3-11d	11-17d	*P*
Water turnover rate (*k*)	0.098 ± 0.0050	0.132 ± 0.0065	0.127 ± 0.0061	0.001*
Intake:				
Total water intake (kg/d)	1.97 ± 0.063	2.80 ± 0.070	2.84 ± 0.075	
Food intake (kg/d)	1.90 ± 0.074	3.31 ± 0.097	3.20 ± 0.086	
Fat intake (kg/d)	0.28 ± 0.011	0.49 ± 0.014	0.48 ± 0.013	
Protein intake (kg/d)	0.18 ± 0.007	0.31 ± 0.009	0.30 ± 0.008	
Energy intake (MJ/d)	15.37 ± 0.597	26.75 ± 0.785	25.88 ± 0.697	
Energy intake (MJ/kg0.75/d)	1.04 ± 0.049	1.80 ± 0.076	1.69 ± 0.073	
Deposition:				
Δ Mass (kg/d)	-0.51 ± 0.065	0.24 ± 0.031	0.14 ± 0.049	<0.001*
Fat deposition (kg/d)	-0.30 ± 0.030	0.02 ± 0.027	-0.11 ± 0.045	0.001*
Protein deposition (kg/d)	-0.04 ± 0.013	0.06 ± 0.009	0.06 ± 0.008	<0.001*

* Significant at the 0.05 level after Bonferroni adjustment for multiple comparisons.

Table 4.12: Mass and body composition of grey seals (n = 10) during Spring 2000B. Values are means ± SE. Mass differences tested by repeated-measures ANOVA. Differences in TBW between initial and final measurements tested by a two-tailed paired t-test.

	Mean	±	SE	*P*	*F*
Mass (kg)					
0d.....	36.1	±	1.90		
3d.....	35.5	±	1.77		
11d.....	37.3	±	1.63		
14/15d..	36.4	±	1.35	0.042	4.39
TBW (kg)					
0d.....	20.8	±	1.04		
3d.....	20.6	±	1.06		
11d.....	21.5	±	1.00		
14/15d..	21.2	±	0.92	0.357	
TBW (%)					
0d.....	57.7	±	0.69		
3d.....	58.1	±	0.60		
11d.....	57.6	±	1.04		
14/15d..	58.2	±	0.98		
TBF (kg)					
0d.....	7.5	±	0.61		
3d.....	7.1	±	0.46		
11d.....	7.8	±	0.66		
14/15d..	7.2	±	0.57		
TBF (%)					
0d.....	20.5	±	1.01		
3d.....	20.0	±	0.88		
11d.....	20.8	±	1.52		
14/15d..	19.8	±	1.43		
TBP (kg)					
0d.....	7.0	±	0.35		
3d.....	7.0	±	0.36		
11d.....	7.2	±	0.35		
14/15d..	7.2	±	0.33		
TBP (%)					
0d.....	19.5	±	0.29		
3d.....	19.6	±	0.25		
11d.....	19.4	±	0.43		
14/15d..	19.7	±	0.41		

* Significant at the 0.05 level after Bonferroni adjustment for multiple comparisons.

Table 4.13: Water, food and energy intake and nutrient deposition during the first, second and third parts of the experimental period of Spring 2000B (n = 10). Values are means ± SE.

	0-3d	3-11d	11-14d	*P*
Water turnover rate (*k*)	0.077 ± 0.0035	0.128 ± 0.0136	0.128 ± 0.0076	0.010*
Intake:				
Total water intake (kg/d)	1.49 ± 0.095	3.02 ± 0.094	2.69 ± 0.150	
Food intake (kg/d)	1.54 ± 0.100	3.60 ± 0.097	2.86 ± 0.283	
Fat intake (kg/d)	0.21 ± 0.014	0.47 ± 0.013	0.38 ± 0.037	
Protein intake (kg/d)	0.15 ± 0.010	0.35 ± 0.010	0.28 ± 0.028	
Energy intake (MJ/d)	11.55 ± 0.753	26.93 ± 0.728	21.39 ± 2.119	
Energy intake (MJ/kg0.75/d)	0.81 ± 0.066	1.80 ± 0.060	1.46 ± 0.156	
Deposition:				
Δ Mass (kg/d)	-0.24 ± 0.108	0.23 ± 0.041	-0.29 ± 0.135	0.003*
Fat deposition (kg/d)	-0.13 ± 0.111	0.08 ± 0.056	-0.19 ± 0.188	0.157
Protein deposition (kg/d)	-0.02 ± 0.025	0.04 ± 0.015	-0.02 ± 0.046	0.236

* Significant at the 0.05 level after Bonferroni adjustment for multiple comparisons.

Maintenance Energy Requirements

Considering the difficulties (and abnormalities) created by the unsuitable diet used in Spring 1999, data from these two experiments were not used to calculate the maintenance energy requirements of 5-month old grey seals. There was a significant relationship between the gross energy intake per metabolic size and mass change (Figure 4.1). The maintenance energy requirement of 5-month old seals averaged 1.56 MJ * $kg^{-0.75}$ * d^{-1}. There was also a significant relationship between gross energy intake per metabolic size and mass change for the 10-month old grey seals (Figure 4.2). The maintenance energy requirement for these animals was 0.95 MJ * $kg^{-0.75}$ * d^{-1}.

There was no relationship between fat deposition rate and gross energy intake per metabolic size in either the 5-month old (Figure 4.3A) or 10-month old (Figure 4.4A) seals. There was also no relationship between fat deposition and fat intake for either group of seals (Figures 4.3B and 4.4B). Protein deposition, however, was directly related to both gross energy intake per metabolic size and protein intake in 5-month old seals (Figures 4.3C and 4.3D) but not in 10-month old seals (Figures 4.4C and 4.4D).

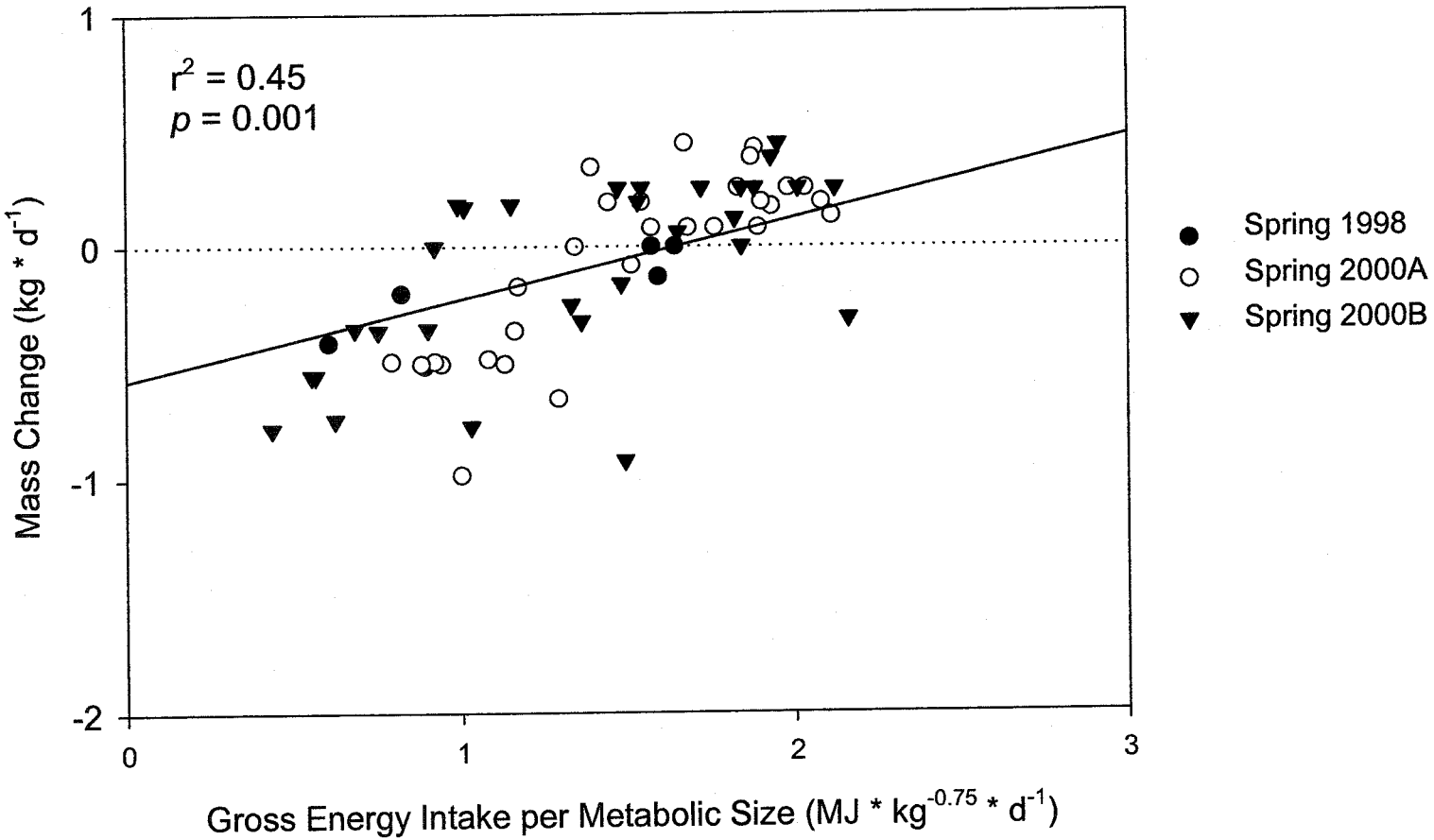

Figure 4.1: Daily mass change as a function of gross energy intake per metabolic body size for 5-month old grey seals. Data represent three time periods of measurement for each individual: Spring 1998 (0-5 d, 5-10 d, 10-13 d); Spring 2000A (0-3 d, 3-11 d, 11-17 d); Spring 2000B (0-3 d, 3-11 d, 11-15 d). Regression relationships and lines plotted were determined from one overall average (0 d to end) for each individual (n=23).

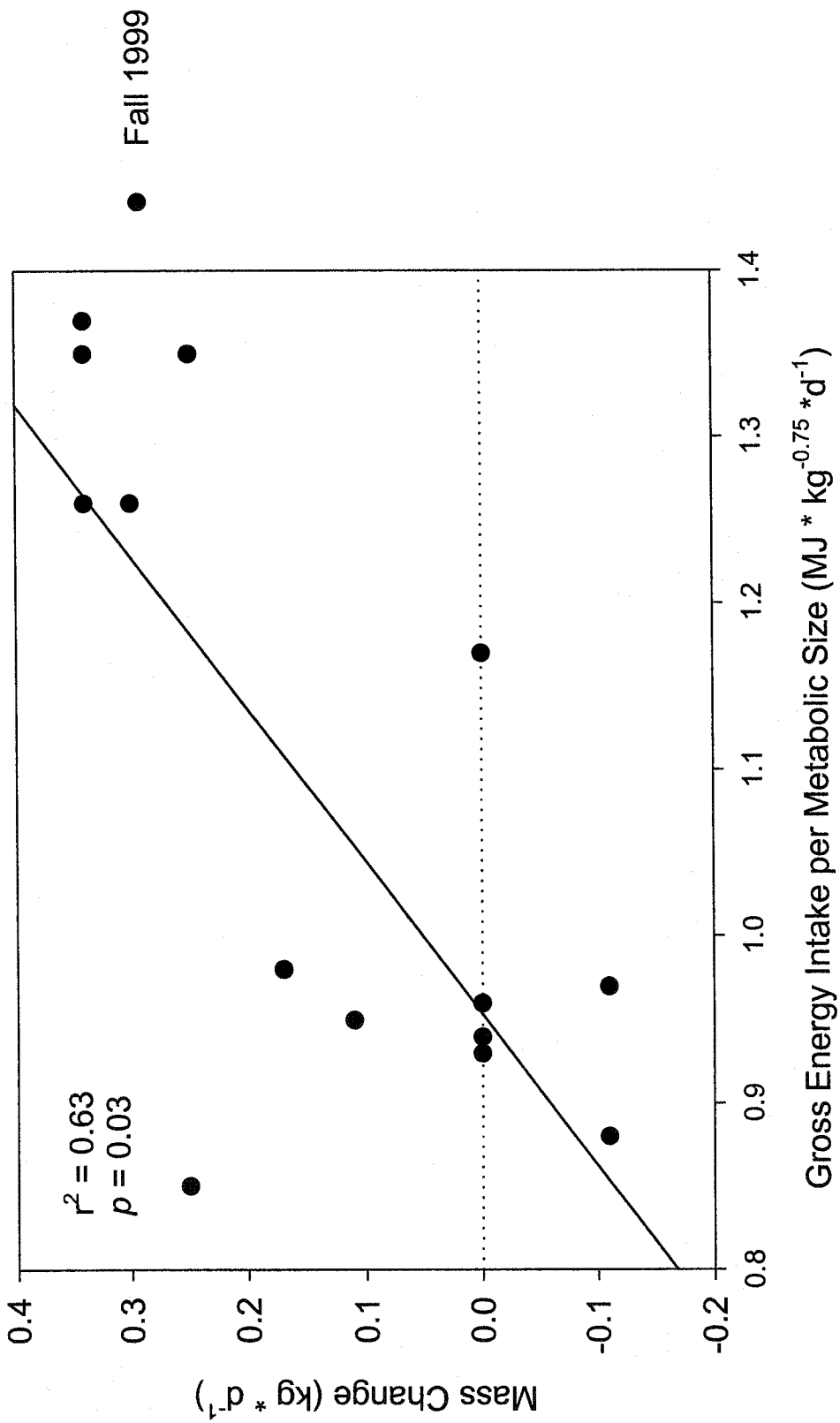

Figure 4.2: Daily mass change as a function of gross energy intake per metabolic body size for 10-month old grey seals. Data represent two time periods of measurement for each individual: 0-9 d and 9-15 d. Regression relationships and lines plotted were determined from one overall average (0-15 d) for each individual (n = 7).

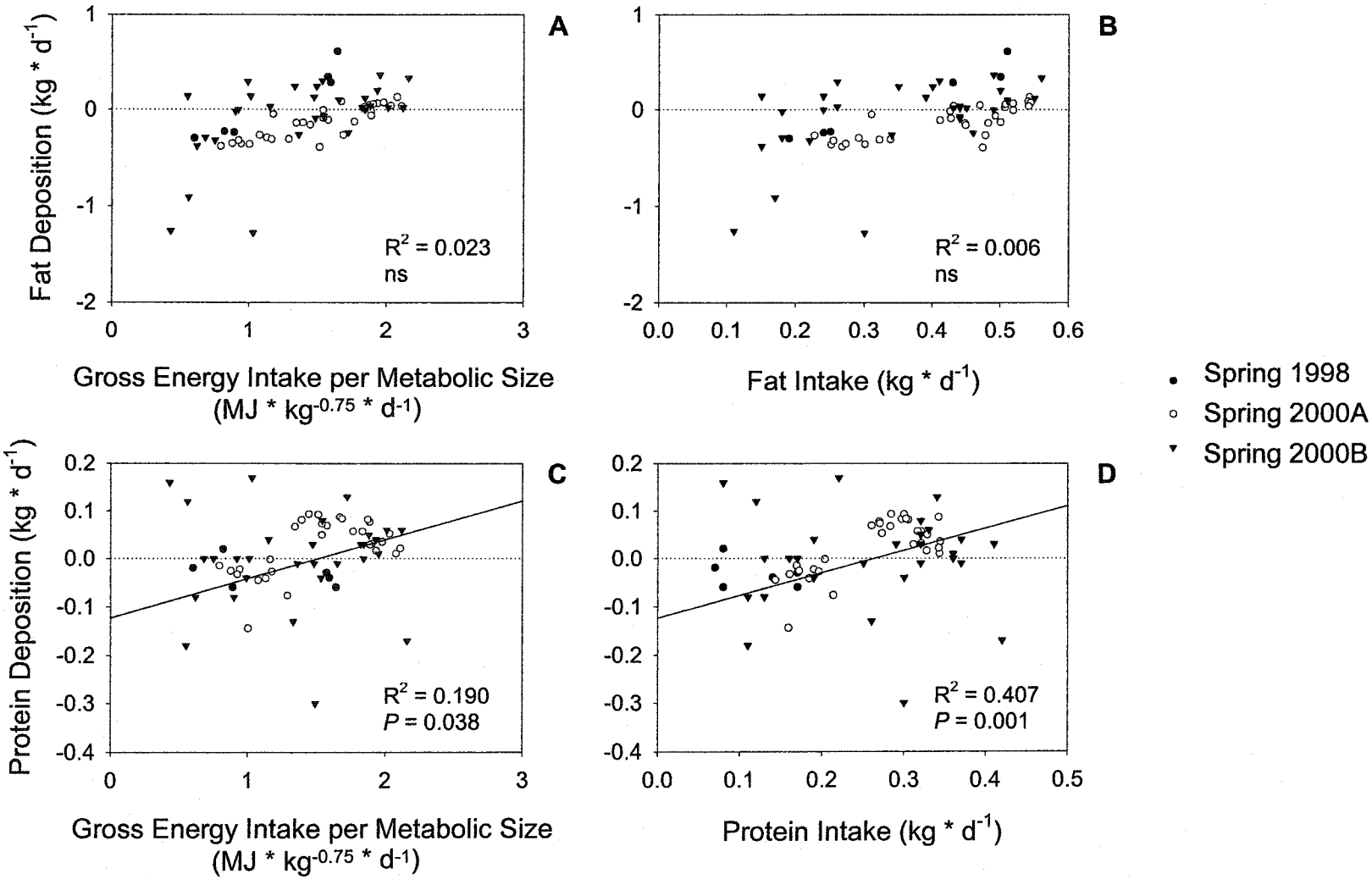

Figure 4.3: Daily fat deposition in 5-month old seals as a function of (A) daily energy intake and (B) daily fat intake, and daily protein deposition in seals as a function of (C) daily energy intake and (D) daily protein intake. Data represent three time periods of measurement for each individual: Spring 1998 (0-5 d, 5-10 d, 10-13 d); Spring 2000A (0-3 d, 3-11 d, 11-17 d); Spring 2000B (0-3 d, 3-11 d, 11-15 d). Regression relationships and lines plotted were determined from one overall average (0 d to end) for each individual (i.e. n = 23).

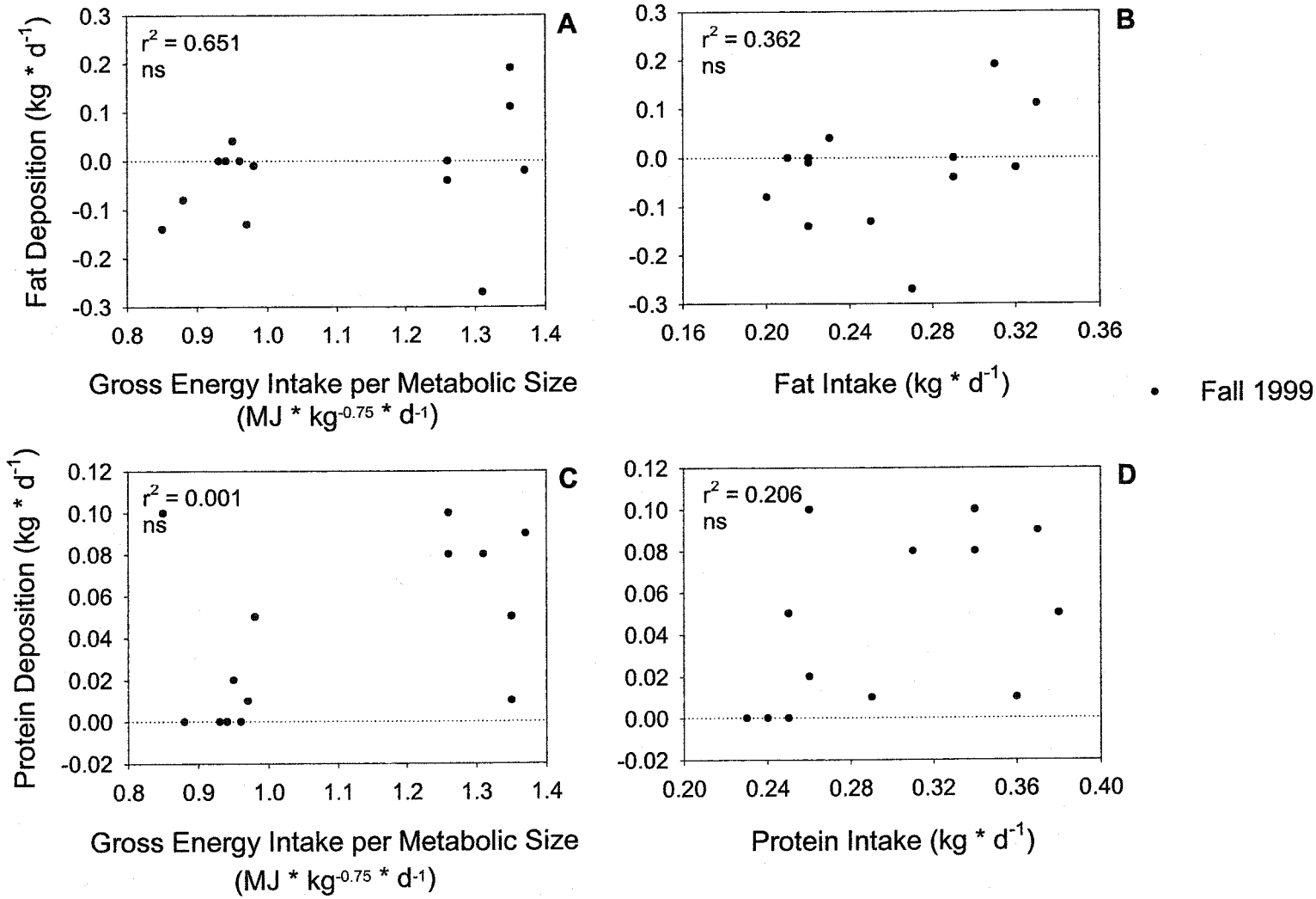

Figure 4.4: Daily fat deposition in 10-month old seals as a function of (A) daily energy intake and (B) daily fat intake, and protein deposition in seals as a function of (C) daily energy intake and (D) daily protein intake. Data represent two time periods of measurement for each individual: 0-9 d and 9-15 d. Regression relationships and lines plotted were determined from one overall average (0-15 d) for each individual (n = 8).

Discussion

Upon completion of the Spring 1999 experiments, consultation with Moore Clark, Inc. revealed that the high level of unidentified material in the diets was due to the presence of carbohydrate. Although we did not know prior to the feeding trials, certain carbohydrates are used as "binders" in the pelleting process for fish-meals. Pinnipeds, like most carnivores, are unaccustomed (and ill-adapted) to consuming carbohydrate. This high level of carbohydrate was most likely responsible for the development of clostridial enteritis in at least several of the seals and in the poor performance of all of the seals in these experiments. *Clostridium perfringens* is an anaerobic Gram-positive bacillus that is naturally present in the intestines of many mammals. Strains of these bacteria are pathogenic by virtue of their ability to produce various toxins. *C. perfringens* can cause a highly fatal enteritis, known as clostridial enteritis, purple gut or enterotoxemia, in livestock animals including sheep, piglets and calves (Lulov and Angelov 1986, Schultz 1994, Taylor 1999, Lewis 2000). Under normal conditions, toxins produced by the bacteria are inactivated by body defence mechanisms. The disease occurs when conditions in the small intestine allow the growth of large numbers of the bacteria. These conditions include an abundance of nutrients, especially carbohydrates, and at least a partial slowdown or stoppage of intestinal tract movement brought about by ingesting a particularly large amount of feed, allowing the toxins to accumulate and be absorbed in the gut. Olsen *et al.* (1996) showed that the stomach capacity of harp seals averaged 4.3% body mass but ranged between 2.1% and 5.8%. Rosen *et al.* (2000) fed juvenile Steller sea lions meals that ranged in size from 0.75% to

4.5% body mass with no adverse effects. The meal size in both of the Spring 1999 experiments was approximately 3.3% body mass and, thus, likely below the animals' stomach capacity and well within the range of meal sizes used by Rosen *et al.* (2000). Abrupt diet changes also affect the ability of normal bacteria to adapt to changing conditions, disrupting the balance of intestinal bacteria. Unfortunately, at least two out of these three conditions likely existed for the seals participating in the Spring 1999 experiments, including those that completed the experiments. Because the health and normal digestion processes of these seals were likely compromised, no further discussion of the results from these experiments can be made.

The water turnover rate was significantly lower in the initial period of each experiment because full rations were not fed until 4 d to allow for a gradual adjustment by the seals to the new diet. Blood sampling at the transition between partial and full rations was not done until the experiments performed in Spring 2000. Thus, values for food, nutrient and energy intakes measured during the later period(s) of each experiment are more representative of intakes once the animals were on full rations. I had estimated that the gross energy intake required for maintenance would be approximately 13.7 MJ/d. This level of intake was not met in the initial period of the majority of the experiments but was exceeded in the full ration periods of all experiments. Despite having intakes in excess of the predicted maintenance requirement, a high rate of mass loss was recorded in the third period of Spring 2000B (Table 4.13). The animals began to show signs of ill health (lethargy, diarrhoea) towards the end of this experiment, which is why the experiment was ended prior to the completion of two weeks on full rations. The mass

loss recorded supports our observation that these animals may also have been experiencing an adverse health reaction to the diet and/or feeding method.

The homogenized diets generally had lower concentrations of water and protein than fish species used in other captive feeding studies (Table 4.1 vs. Table 4.14). The highest moisture content in any of my experimental diets was approximately 73% (Fall 1999), which is at the low end of the moisture contents found in whole fish (usually 75-79%). The highest protein content in the non-pellet based diets was only 11.1% (Fall 1999), which is lower than any of the protein contents found for the whole fish studied (Table 4.14). It is also lower than the 15% protein diet that I was attempting to manufacture. The protein contents of the diets could not be determined prior to the experiments as the homogenous diets were prepared in the field. Rather they were estimated based on the predicted protein content of the fish used, which were obviously underestimated. The fat contents of my experimental diets, on the other hand, were all higher than those found for the fish presented in Table 4.14. This is not to say that my experimental diets were higher in fat than all fish species, particularly at certain times of the year. Lawson *et al.* (1997) fed harp seals capelin that were 19.2% fat and pacific herring and eulachon (*Thaleichthys pacificus*) can both exceed 20% fat in the spring and summer (Iverson *et al.* 2002). However, the ratio of fat to protein may be a more critical issue, as it is usually <0.6 and only exceeds this in the highest fat eulachon at ~1.5 (Table 4.14, Payne *et al.* 1999). In contrast, diets used in my studies had ratios ranging from 0.7 to 2.9. Thus, a diet with a very high fat to protein ratio may be difficult to assimilate.

The efficiency with which animals digest their food varies depending on factors such as food quality (energy and lipid content), meal size, time between meals, digestive

Table 4.14: Proximate composition (w/w) and energy content of several fish species used in captive feeding studies

Species	Fat %	Protein %	Water %	Energy (MJ/kg)	Source
Capelin (*Mallotus villosus*)	3	16	78	4.5	Brekke and Gabrielsen 1994
Arctic Cod (*Boreogadus saida*)	5	15	77	4.9	Brekke and Gabrielsen 1994
Herring (*Clupea harengus*)	9.1	17.2	72.4	8.24	Rosen and Trites 1999
Squid (*Loligo opalescens*)	2.4	12.8	82.5	3.69	Rosen and Trites 1999
Salmon (*Oncorhynchus gorbuscha*)	2.6		74.7	5.21	Rosen and Trites 2000
Pollock (*Theragra chalcogramma*)	0.8	19.2	78.8	4.55	Ashwell-Erickson and Elsner 1981
Mullet (*Liza richardsoni*)	2.7	18.3	73.7	4.25	Heath and Randall 1985
Anchovy (*Engraulis capensis*)	7.0	14.1	75.0	5.56	Heath and Randall 1985
Squid (*Loligo reynaudi*)	1.9	15.4	78.9	4.55	Heath and Randall 1985
Shrimp (*Pandalus borealis*)	5.6	12.2	73.2	5.09	Keiver et al. 1984
Flagtail (*Kuhlia sandvicensis*)	5.3	18.2	73.3	6.40	Goodman-Lowe et al. 1999
Lobster (*Panulirus marginatus*)	0.2	21.2	76.2	5.06	Goodman-Lowe et al. 1999

tract morphology, and nutritional state (Golley *et al.* 1965, Grove *et al.* 1985, Heath and Randall 1985, Jackson 1986, Brekke and Gabrielsen 1994, Lawson *et al.* 1997a,b Goodman-Lowe *et al.* 1999, Rosen and Trites 2000a, Rosen *et al.* 2000). Digestible energy (DE) is the proportion of ingested energy absorbed from the gastrointestinal tract. Metabolizable energy (ME) is the proportion of ingested energy that is available for maintenance functions, growth, reproduction and external work and may be a more direct measure of the benefit that animals derive from their diet (Rosen and Trites 2000a). Both DE and ME values are high in pinnipeds (Miller 1978, Ashwell-Erickson and Elsner 1981, Keiver *et al.* 1984, Ronald *et al.* 1984, Fadely *et al.* 1990, Fadely *et al.* 1994, Mårtensson *et al.* 1994, Lawson *et al.* 1997a,b, Rosen and Trites 2000a). This is somewhat surprising considering the rapid rate of passage of digesta reported for pinnipeds (Eastman and Coalson 1974, Helm 1983, Helm 1984, Markussen 1993) relative to other carnivores (Warner 1981) but may be explained by the much longer small intestine of pinnipeds compared to other carnivores (King 1964, Bonner 1981, Helm 1983, Olsen *et al.* 1996).

DE and ME in pinnipeds are positively correlated with the prey lipid content or energy density (Miller 1978, Keiver *et al.* 1984, Fadely *et al.* 1994, Mårtensson *et al.* 1994, Lawson *et al.* 1997a,b, Rosen and Trites 2000a). It appears that pinnipeds can assimilate diets with very high lipid contents. Lawson *et al.* (1997b) found the relationship between DE and prey energy density to be linear as opposed to curvilinear; no asymptote was apparent even when diets of 19% fat, with a fat to protein ratio of 1.4, were fed to captive harp seals. Lawson *et al.* (1997a,b) did, however, find that both ringed seals and harp seals required an acclimation period, for the DE to reach

equilibrium, when they were switched to these high fat diets. The animals' faeces were extremely oily during the first week of the capelin trials but fat content decreased significantly in the second week (Lawson *et al.* 1997b). They proposed that the intestinal mucosa requires time to develop sufficient surface area necessary to absorb the greater lipid content of high fat prey (Karasov and Diamond 1983, Gross *et al.* 1985, Lloyd *et al.* 1994).

Although DE and ME were not calculated in the experiments described herein, there was some evidence, from faecal fat contents, that the diets were under-utilized. Keiver *et al.* (1984) found only trace amounts of neutral lipid in faeces, values too small to be distinguished from analytical error. These faecal lipid losses were unaffected by total lipid intake and they suggested that faecal lipid was primarily endogenous in origin as had been reported for other carnivores (Schneider and Flatt 1975). Ronald *et al.* (1984) showed similar results up to total lipid intakes of approximately 0.55 kg/day. The highest total lipid intake in any of my experiments occurred during Spring 2000A and was lower than those of Ronald *et al.* (1984), at 0.49 ± 0.014 kg/day. Thus, at this level of lipid intake I expected the faecal lipid losses to be negligible and the amount of lipid in the faeces to be very small. Faecal samples were collected on 12 d of the experiment to avoid measuring potentially inflated lipid contents associated with an initial acclimation period (Lawson *et al.* 1997a,b). Despite this, the average lipid content of the faeces collected was 4.7% ranging as high as 14.6%. This implies that there were significant dietary contributions to faecal lipid and that the animals were not able to absorb all the fat presented to them in their diet.

The apparent inability of the seals to fully digest the experimental diets may be partially a consequence of the feeding method used. In order for the feed to pass through the stomach tube, it had to have the consistency of a viscous liquid. A finely ground or more liquid diet has a reduced retention time (Clemens *et al.* 1975, Jenkins *et al.* 1978, Leeds *et al.* 1979) and lower digestibility relative to a more fibrous or solid diet (Blaxter *et al.* 1956, Larsen *et al.* 1994). Also, the majority of the total fat in the diet was added in liquid form as opposed to being a part of the fish tissue. Supplementing a diet with fat reduces the efficiency of energy utilization (Harris 1991, Iossa *et al.* 1997, Donnelly *et al.* 2003). This may, in part, be because the fluids and soft items in the digesta are the first to be transported through the digestive system (Clemens *et al.* 1975, Treacy and Crawford 1981, Markussen 1993). Thus, the essentially liquid nature of the diets fed to the seals, particularly their fat component, may have reduced the animals' ability to absorb nutrients from them.

Because I had no data on the digestive efficiency of the experimental diets I did not calculate ME. Instead, I determined maintenance energy requirements based on GEI. The maintenance energy requirement of 5-month old grey seals was estimated to be 1.56 MJ GEI * $kg^{-0.75}$ * d^{-1} (Figure 4.1) and that of 10-month old grey seals was 0.95 MJ GEI * $kg^{-0.75}$ * d^{-1} (Figure 4.2). Kirsch *et al.* (2000) found the maintenance energy requirement of juvenile (1-3 yr) harp seals consuming a low-fat diet to be 2.0 MJ GEI * $kg^{-0.75}$ * d^{-1}. Estimates of the maintenance energy requirement of harp and grey seals reported in Keiver *et al.* (1984) and Ronald *et al.* (1984), respectively, are expressed in terms of ME. Assuming that ME is approximately 85% of gross energy intake, which is in line with results for pinnipeds consuming various prey (Keiver *et al.* 1984, Ronald *et*

al. 1984, Mårtensson *et al.* 1994, Lawson *et al.* 1997a,b, Rosen and Trites 2000a), I was able to express their findings in terms of GEI. Keiver *et al.* (1984) found maintenance energy requirements of 15 month old harp seals to be 0.62 MJ GEI * $kg^{-0.75}$ * d^{-1}. Ronald *et al.* (1984) found maintenance energy requirements of 0.78, 0.74 and 0.44 MJ GEI * $kg^{-0.75}$ * d^{-1} for 1 year-old, 2 year-old and adult grey seals, respectively. The large variation in these estimates is likely due to a combination of the ages of the study animals and the digestibility of their diets.

Immature mammals are expected to require approximately twice Kleiber's predicted maintenance energy for adult mammals as a result of their increased energy expenditures associated with growth (Kleiber 1975). Because maintenance energy requirements decline with age, some part of the difference found between 5-month and 10-month old seals may be a result of their age differences. The lower values reported by Keiver *et al.* (1984) and Ronald *et al.* (1984) may also relate to the older age of their study animals compared to mine. Age, however, cannot explain the relatively larger value reported by Kirsch *et al.* (2000) for harp seals consuming a low-fat diet. Low-fat diets are associated with reduced ME (Keiver *et al.* 1984, Mårtensson *et al.* 1994, Lawson *et al.* 1997a,b, Rosen and Trites 2000a) and increased energy losses (e.g. HIF Rosen and Trites 1997, 2000b). Thus, if the ME of the diet used in Kirsch *et al.* (2000) was less than 85%, their results would not be directly comparable to those I calculated from Keiver *et al.* (1984) and Ronald *et al.* (1984) and would appear inflated. The problems associated with the digestibility of my experimental diets also likely contributes to the differences in the maintenance energy requirements reported herein and those calculated from Keiver *et al.* (1984) and Ronald *et al.* (1984). This conclusion is

supported by the fact that when I calculated maintenance energy requirements for the Spring 1998 animals alone the value was 3.7 MJ GEI * $kg^{-0.75}$ * d^{-1}. The Spring 1998 animals consumed the highest-fat diet, with the highest fat to protein ratio, so an inability to completely utilize dietary fat may have inflated the estimate of gross maintenance energy requirement in this experiment more than in the others. This may also help to explain some of the difference between the 5-month and 10-month old seals' maintenance energy requirements. The 10-month old seals consumed a lower fat diet with a lower fat to protein ratio (0.9) than did any of the 5-month old seals such that there may not have been as much of a problem with the digestibility of that diet.

No relationship was found between fat deposition rate and either gross energy intake per metabolic size (Figures 4.3A and 4.4A) or fat intake (Figures 4.3B and 4.4B) in either the 5-month old or 10-month old seals. Protein deposition, however, was directly related to both gross energy intake per metabolic size and protein intake in the 5-month old seals (Figures 4.3C and 4.3D). The lack of similar findings in the 10-month old seals may be due to the smaller sample size (Figures 4.4C and 4.4D). The lack of a relationship between fat intake and deposition likely arises from the seals' apparent inability to completely digest and absorb the dietary fat. The correlation between gross energy intake per metabolic size and protein deposition but not fat deposition may be related to the young age of the animals studied. It appears that these animals preferentially utilized their dietary energy for the development of lean body mass, which in young, rapidly growing animals takes priority over the accumulation of fat stores.

Dietary protein requirements for early growth in weanling mammals vary depending on the species (Robbins 1993). Young cats require very high protein

concentrations in their diets (33%, Scott 1968). Other young carnivores such as mink and foxes need protein concentrations of approximately 25% (NRC 1968) while white-tailed deer (*Odocoileus virginianus*) require levels between 13-20% (French *et al.* 1956, McEwen *et al.* 1957). The dietary protein requirements of adult mammals are lower than for rapidly growing juveniles and they can range from 19-25% for adult carnivores to as low as 5-9% for wild ruminants (Robbins 1993). Thus, the dietary protein levels supplied in my experimental diets (< 12%, Table 4.1) appear to have been sub-optimal, especially in relation to the fat content fed.

The diet used in Spring 1998 had the lowest protein content (8.3 ± 0.61%, Table 4.1) which led to the lowest daily protein intakes in any of the experiments (Table 4.3). The animals fed this diet appear to have lacked sufficient protein intakes since protein, as a proportion of body mass, declined over the course of the experiment (Table 4.2). Interestingly, despite a loss of body mass throughout the experiment, total body fat increased both absolutely and in proportion to body mass (Table 4.2). Similar results were found in young grizzly bears (*Ursus arctos horribilis*) fed high-energy, low-protein diets (Felicetti *et al.* 2003); at, as well as slightly below, mass maintenance bears gained fat mass while losing lean body mass. In other words, energy was accumulated at mass maintenance. Wakshlag *et al.* (2003) also found that low protein diets can lead to lean body wasting while augmenting the accumulation of adipose tissue in adult canines.

The Fall 1999 and Spring 2000 diets had higher protein concentrations than the Spring 1998 diet (Table 4.1) and the animals fed these diets had higher daily protein intakes (Tables 4.9, 4.11 and 4.13). Generally speaking, when the daily protein intakes were greater than 0.26 kg/d, seals were able to maintain their total body protein stores

(Figures 4.3D and 4.4D). This is equivalent to approximately 7 g/kg/d, which is much higher than the less than 2 g/kg/d digestible protein intake necessary for maintenance of lean body mass in grizzly bears (Felicetti *et al.* 2003). This difference may arise from the fact that seals are more obligate as carnivores and that, as omnivores, bears may have developed a relatively more efficient utilization of protein (Robbins 1993). Alternatively, the difference may stem from the fact that I measured total protein intake and Felicetti *et al.* (2003) measured digestible protein intake. Again, depending on the digestibility of the diets used in my experiments, these values may not be all that different.

Considering the adverse health effects exhibited by many of the seals, and the apparently low utilization of the energy in the homogenous diets, this feeding method has proven less than ideal and I would not recommend its application in future studies. It seemed an obvious way to circumvent the issues of maintaining seals in captivity for long periods while also allowing the feeding of completely homogenous diets. It was simply not possible to deliver enough energy in an adequately proportioned and digestible diet by stomach intubation, without increased numbers of feeding per day. As it was, the stomach intubation procedures were extremely labour intensive and somewhat stressful to the seals. Despite the difficulties encountered, it is possible to make several conclusions. First, provided there are sufficient protein intakes, the daily energy budget of young grey seals appears to be primarily partitioned into maintenance and growth of lean body mass, as would be expected. However, it is likely that there is a greater potential for simultaneous fattening (with more suitable diets) than was found in these experiments, given the presence of many young of the year on Sable Island that have ample fat stores (pers. observations). Second, although the absolute values of gross maintenance energy

requirement reported in this study are likely overestimates, it appears that maintenance energy requirement declines with age within the first year of life. Finally, unlike Lawson *et al.* (1997b) who found a linear relationship between DE and prey energy density, there appears to be a limit to the seals' ability to assimilate diets with very high lipid contents when diets are supplemented with lipid in a liquid form.

Chapter 5. Quantitative Fatty Acid Signature Analysis in Juvenile Grey Seals

Introduction

Quantitative FA signature analysis (QFASA, Iverson *et al.* 2004) is based on the principle that FA in prey species are transferred, largely unaltered or at least in a predictable manner, to lipid storage sites (i.e. adipose tissue) of their predators. Each prey species tends to have a unique pattern of FA, owing to its own life history and dietary habits, which acts like a signature or fingerprint. Because these signatures are conserved through the food chain, they can act as indicators of diet composition. QFASA has several potential advantages over traditional methods used to estimate predator diets. First, the use of FA does not depend on the recovery of prey hard parts such that a greater variety of prey species can be identified as contributing to the diet. Second, because QFASA provides information on individual animals and since the sampling methods are non-lethal, this method can be used to study diet longitudinally and ask questions at the level of both individuals and populations. Finally, while QFASA may not be able to identify prey items consumed only occasionally it can determine the prey that the predator relies on for survival and does so on a time scale relevant to the animals' ecology. The QFASA model, developed by Iverson *et al.* (2004), uses the signatures of all possible prey species to compute the mixture of signatures (species and proportions of consumption) that most closely resembles that of the predator. QFASA provides, for the first time, quantitative estimates of the proportions of different prey species in the diets of individual predators.

In addition to a detailed knowledge of the FA signatures of all potential prey, an understanding of the biochemistry and metabolism of FA within predators is essential to being able to use this method accurately and with confidence. Currently, two important parameters have been incorporated into the QFASA model to account for these metabolic considerations: the set of calibration coefficients for individual FA and the subset of FA used in modeling diets (Iverson *et al.* 2004). To date, these two issues have received only preliminary consideration, which has indicated their importance. Controlled feeding studies are necessary for the further determination and optimization of these parameters.

Calibration coefficients have been developed from feeding studies of individuals on a given long-term diet, with the assumption that after such long-term feeding, the predator's adipose tissue stores will resemble diet as much as it ever would. The calibration coefficient is then calculated as the discrepancy, or ratio, between adipose tissue and diet levels of each FA (Iverson *et al.* 2004). Thus, calibration coefficients are mathematical representations of the relationships between dietary and adipose tissue FA and provide a composite picture of the influence of the differential metabolism of individual FA on the overall FA composition of predator adipose stores. Calibration coefficients are included in the model by replacing the predator's observed proportion of a specific FA with the calibrated proportion of that FA. This produces the adipose tissue FA composition that would be expected if no differential FA metabolism was occurring within the predator. It is this FA signature against which the mixture of prey signatures is then optimized. Although calibration coefficients derived from different experiments show relatively consistent patterns of FA metabolism, their specific values can vary depending on predator species and lipid content of the diet (Iverson *et al.* 2004). It is,

therefore, important to determine the factors which have the greatest influence on these coefficients, in order to assess the most applicable set for a given study species.

The second issue, as stated above, relates to the FA subset used in assessing diets. Marine lipids contain a large array of FA and approximately 70 of them are routinely identified using gas chromatography and a polar capillary column. Since certain FA originate from either endogenous or exogenous sources while others are a result of contributions from both, individual FA vary in the degree to which they reflect diet (see Chapters 1 and 2). This raises the question of whether to estimate the diet using all FA identified or some subset of these FA. Iverson *et al.* (2004) automatically excluded from use those FA that are entirely endogenous in origin as well as those that are generally found in trace amounts and are difficult to identify reliably. The two subsets of FA investigated by Iverson *et al.* (2004) were a so-called "dietary" set and an "extended-dietary" set. The former consisted of FA that are strictly dietary in origin while the latter included several FA that can be biosynthesized by the predator but that can also be indicative of dietary intake (Iverson 1993, Budge *et al.* 2002, Iverson *et al.* 2002, Iverson *et al.* 2004). In general, the extended-dietary set performed better in their simulations and modeling exercises, likely due to the increased information provided, but they suggest that fine-tuning of these FA sets is appropriate and that the best FA set may also vary with the predator being studied.

In this study, I conducted controlled feeding experiments designed to investigate some of the issues relating to calibration coefficients, optimization of FA sets and validation of QFASA. The experiments carried out in this study were not long enough to be used to actually calculate calibration coefficients, however, my use of completely

homogenous diets, with known FA compositions, provided a unique opportunity to test model parameters. In previous controlled feeding studies, seals were fed individual fish of a given species and FA were analyzed from a separate sample of fish from the same lot (e.g., Kirsch 1997, Kirsch *et al.* 2000). However, because individual fish of a species can vary substantially in FA composition and fat content (Jangaard 1974, Montevecchi and Piatt 1984, Stansby 1986, Mårtensson *et al.* 1996, Budge *et al.* 2002, Iverson *et al.* 2002), the exact dietary intake of FA was not known. Because I had well-defined dietary FA signatures and *a priori* expectations regarding certain model outcomes, the goal of my experiments was to assess the influence of varying the set of calibration coefficients and FA subset on the accuracy of the QFASA model in a more comprehensive manner than previously possible.

Methods

Animal Maintenance and Sampling

Juvenile grey seals (5-10 months) were captured from the beaches of Sable Island, Nova Scotia, Canada in spring 1998 (n = 4) and 1999 (n = 8 and 8), fall 1999 (n = 8) and spring 2000 (n = 10 and 10). These seals were used in 6 independent experiments. Seals were placed in large, covered pens that were equipped with a fire hose and pump that delivered seawater from the ocean for twice-daily immersion (see Chapter 4). Homogenous diets were prepared daily from ground fish (Spring 1998, Fall 1999, Spring 2000) or pelleted fish meal (Spring 1999), and supplemented with fish oil (Table 5.1). Diets were sub-sampled each day for analysis of fat content and FA composition.

Table 5.1: Type of fish and oil used to make experimental diets, as well as percent fat and protein contents (w/w). Values are mean ± SE.

Experiment	Protein Source	Fat Supplement	Fat %	Protein %
Spring 1998	Ground Herring	Menhaden Oil	24.5 ± 0.69	8.3 ± 0.61
Spring 1999A	Pelleted Fish-Meal	Menhaden Oil	12.0 ± 0.62	12.7 ± 0.83
Spring 1999B	Pelleted Fish-Meal	Menhaden Oil	10.5 ± 0.55	13.8 ± 0.33
Fall 1999	Ground Cod	Anchovy Oil	9.7 ± 0.47	11.1 ± 0.13
Spring 2000A	Ground Cod	Capelin Oil	14.9 ± 0.71	9.4 ± 0.21
Spring 2000B	Ground Cod	Herring Oil	13.2 ± 0.62	9.7 ± 0.16

Before the beginning of each diet trial the seals were fasted overnight. On the first day (0 d), the mass of each seal was measured to the nearest 0.5 kg on a 100-kg Salter scale. A blubber biopsy was then taken from both the left and right flank to establish an initial blubber FA signature for each animal. The area was first shaved and cleaned with prepadine, and a local anaesthetic (3 cc lidocaine) was administered. A small incision (< 1 cm) in the skin was made with a sterile #11 scalpel. A sterile 6-mm biopsy punch was then inserted and a core was taken through the full depth of the blubber layer (about 4 cm). The incision was cleaned with topazone and closed with a single interrupted suture. Each blubber biopsy was divided into inner (the half closest to body core) and outer (the half closest to skin) layers (estimated by eye) and individually wrapped in tinfoil. In the field lab (~1 hr after collection), samples were weighed and transferred to glass sample tubes (with Teflon-lined caps) containing 3 ml of chloroform with 0.01% 2,6-di-*tert*-butyl-4-methyl-phenol (BHT) and stored frozen (-20°C) until lipid extractions were performed (< 6 months later).

After the initial biopsy, seals were restrained using a net and fed a known quantity of the homogenous experimental diet by gastric intubation using a ¾ inch foal stomach tube. Feedings were performed twice daily until the end of each diet trial (13-19 d, see Chapter 4). On the last day of each experiment, a blubber biopsy was again taken from each side of the animal using the above sampling procedures, split into inner and outer layers, and stored frozen in solvent with BHT until analysis.

Laboratory Procedures

Lipids were extracted from each blubber section and all diet samples according to a modified Folch *et al.* (1957) procedure (Iverson *et al.* 2001). Briefly, samples were extracted with 2:1 chloroform:methanol and dried over anhydrous sodium sulphate. FA methyl esters (FAME) were prepared from 100 mg of the pure extracted lipid using 8% boron trifluoride in methanol (v/v) in a modification of Morrison and Smith (1964) as described previously (Iverson *et al.* 1997b). FAME were extracted into hexane, concentrated and brought up to volume (50mg/mL) with high-purity hexane.

Duplicate analyses of FAME were performed using temperature-programmed gas-liquid chromatography, according to Iverson *et al.* (1997b), on a Perkin Elmer Autosystem II Capillary FID gas chromatograph fitted with a 30-m column (0.25-mm inside diameter) coated with 50% cyanopropyl polysiloxane (0.25-μm film thickness; J&W DB-23; Folsom, CA, USA). Identifications of FAs were determined from a number of sources, including known standard mixtures (Nu Check Prep., Elysian, MN, USA), silver-nitrate chromatography and GC-mass spectrometry (Iverson *et al.* 1992, 2002).

Data Analysis

Initial and final FA compositions for each animal were calculated by averaging the left and right samples of the inner and outer blubber layers, respectively. Whole blubber FA compositions were reconstructed from the inner and outer blubber signatures by weighting according to the percent of the total blubber mass that each sample represented. FA composition of the diet was determined for each day of the experiment and an overall average calculated.

To determine whether the blubber FA composition had changed in the expected direction of the dietary FA signature over the course of the feeding experiment, I calculated Kulback-Leibler (K-L) distances between the initial FA composition and the diet signature and between the final FA composition and the diet signature. The average K-L distance represents a measure of the distance between two profiles of proportions and is calculated as follows: $\Sigma_j (I_j - F_j)\log(I_j/F_j)$ where j = individual FA, I = initial blubber profile and F = final blubber profile. Paired t-tests were then performed on the distance measures.

To test whether the observed change in FA composition over the course of an experiment was as expected, I calculated a predicted final whole blubber composition for each animal. To calculate this predicted final blubber composition, I used one simplified scenario that might resemble actual responses. First I considered the blubber to be a single pool and made the assumption that all FA consumed were first deposited into existing blubber fat stores and then FA mobilized for energy were taken from this total pool. I then calculated each seal's expected final FA composition using 1) its initial quantity of body fat (kg) from isotope dilution (see Chapter 4), 2) the initial FA composition of that fat (blubber), 3) its daily metabolizable fat intake (as measured by D_2O turnover and assuming an overall 85% metabolizable energy of the diet), 4) the FA composition of the diet fed, 5) calibration coefficients (CC) published in Iverson *et al.* (2004), and 6) the estimated daily rate of fat deposition (also measured by D_2O dilution). The following formula was used:

$$(FA_j)_t = \frac{[(\text{Fat Mass})_t * (FA_j)_{t-1} + (\text{Metabolizable Fat Intake})_t * ((FA_j)_{\text{Diet}} * CC_j)]}{[(\text{Fat Mass})_t + (\text{Metabolizable Fat Intake})_t]}$$

where $(\text{Fat Mass})_t = (\text{Fat Mass})_{t-1} + (\text{Fat Deposition})_{t-1}$. This calculation was iterated for each day of the experiment such that each day started with the estimated blubber FA composition of the previous day.

It is likely that some proportion of the metabolizable fat was used directly for energy rather than first deposited in the adipose tissue. Thus, expected final FA compositions representing an assumed maximum and minimum possible incorporation of the experimental diet were calculated by setting the deposition rate of the metabolizable fat intake at 100% and 20%, respectively. The 20% value was chosen for the minimum incorporation of the dietary FA because Kirsch *et al.* (2000) showed that estimated final FA compositions based on deposition rates below this were unlikely.

Previous experiments have indicated that the inner layer of the blubber responds to a change in diet more strongly than the outer or whole blubber (Kirsh *et al.* 2000, Cooper *et al.* 2001). To estimate final FA compositions based on inner blubber samples, for each day of feeding, a new inner blubber FA composition was estimated based on the assumption that the active FA pool consisted of just the inner blubber layer. Therefore, dietary FA that were deposited each day were only deposited in the inner blubber layer and any FA that were mobilized from the adipose tissue were also taken from this inner blubber layer FA pool. The formula used in these calculations was identical to the above except that the estimated mass of the inner blubber layer was used in the fat mass term.

Initial and final blubber FA compositions (for both whole and inner blubber samples) were used to estimate the prey species composition of the diet of each animal using the QFASA model of Iverson *et al.* (2004). All prey FA signatures used in this study, except those of the experimental diets, were taken from a FA database of prey

collected from the Scotian Shelf, which consists of 1672 individuals of 25 fish and invertebrate species (Budge *et al.* 2002 and unpublished data; Table 5.2). The primary purpose of this was to determine whether the QFASA model was able to identify the experimental diets as contributing to the blubber signatures after only two weeks of feeding. In addition, because the experimental diets should not have been present in the initial signatures, I could use the QFASA estimates of the initial diets to optimize two important sets of parameters used in the OFASA model: the set of calibration coefficients and the subset of FA.

Three sets of calibration coefficients that have been empirically generated are presented in Iverson *et al.* (2004). Two sets are based on juvenile grey or harp seals that were fed a fish diet and one set is based on grey seal pups consuming milk. While the latter experiment was the most controlled, the very high fat content of the milk diet and the much more direct deposition of FA in the pup blubber may make these calibration coefficients less representative of my study animals. Thus, the calibration coefficients that I tested here were the average of those generated in the grey and harp seal experiments (Grey.Harp.CC) and the average of all three sets (Grey.Harp.Pup.CC) (Table 5.3). To determine which of the "extended-dietary" FA, namely 14:0 (A), 18:0 (B), 18:1n-7 (C) and 18:1n-9 (D), to use in the model, I tested the dietary FA set alone and with all possible combinations of this extended set (Table 5.4).

Diet estimates were then generated from each blubber sample, using the various model parameters, according to Iverson *et al.* (2004). The sets of parameters were assessed based on three different criteria: 1) minimization of the false positive identification of the experimental diet in the initial model prediction, 2) goodness of fit in

Table 5.2: Species name and sample size of fish and invertebrates collected from the Scotian Shelf included in the prey fatty acid database.

Common Name	Scientific Name	N
Flounders		
American Plaice	*Hippoglossides platessoid*	125
Windowpane Flounder	*Scophthalmus aquosus*	12
Winter Flounder	*Pseudopleuronectes americanus*	25
Witch Flounder	*Glyptocephalus cynoglossus*	24
Yellowtail Flounder	*Limanda furruginea*	136
Forage Fish		
Capelin	*Mallotus villosus*	132
Gaspereau	*Alosa pseudoharengus*	48
Herring	*Clupea harengus*	196
Mackerel	*Scomber scombrus*	34
Northern Sandlance	*Ammodytes dubius*	120
Snake Blenny	*Lumpenus lumpretaeformis*	18
Gadids		
Cod	*Gadus morhua*	118
Haddock	*Melanogrammus aeglefinus*	142
Pollock	*Pollachius virens*	53
Red Hake	*Urophycis chuss*	38
Silver Hake	*Merluccius bilinearus*	58
White Hake	*Urophycis tenuis*	50
Skates		
Thorny Skate	*Raja radiata*	57
Winter Skate	*Raja ocellata*	15
Other Fish		
Longhorn Sculpin	*Myoxocephalus octodecemspinosus*	45
Ocean Pout	*Macrozoarces americanus*	18
Redfish	*Sebastes sp.*	66
Invertebrates		
Lobster	*Homarus americanus*	9
Northern Shrimp	*Pandalus borealis*	96
Rock Crab	*Cancer irroratus*	37
Total		**1672**

Table 5.3: Calibration Coefficients derived from Iverson et al. (2004) tested as model parameters.

Fatty Acids	Calibration Coefficient		Fatty Acids	Calibration Coefficient	
	Grey.Harp	Grey.Harp.Pup		Grey.Harp	Grey.Harp.Pup
12:0	0.91	0.92	18:1n-5	1.02	1.01
13:0	1.00	1.00	18:2d5,11	1.02	0.97
Iso14	1.00	1.00	18:2n-7	1.07	1.13
14:0	0.90	0.91	18:2n-6	1.80	1.55
14:1n-9	0.88	0.83	18:2n-4	0.92	0.92
14:1n-7	1.08	1.14	18:3n-6	1.01	0.93
14:1n-5	9.88	7.10	18:3n-4	2.45	1.97
Iso15	1.11	1.05	18:3n-3	1.87	1.61
Anti15	1.12	1.03	18:3n-1	0.95	0.93
15:0	1.03	1.01	18:4n-3	0.98	0.97
15:1n-8	1.00	1.00	18:4n-1	1.25	1.17
15:1n-6	1.12	1.15	20:0	0.50	0.67
Iso16	0.99	0.98	20:1n-11	3.13	2.41
16:0	0.69	0.74	20:1n-9	0.90	0.91
16:1n-11	2.38	1.91	20:1n-7	0.88	0.86
16:1n-9	3.01	2.38	20:2n-6	1.52	1.35
16:1n-7	1.57	1.48	20:3n-6	1.04	0.99
7Me16:0	1.09	1.07	20:4n-6	0.93	0.93
16:1n-5	1.09	1.06	20:3n-3	1.07	1.04
16:2n-6	0.75	0.77	20:4n-3	1.81	1.54
Iso17	1.07	1.03	20:5n-3	0.73	0.76
16:2n-4	1.23	1.11	22:1n-11	0.27	0.34
16:3n-6	0.99	0.99	22:1n-9	0.43	0.45
17:0	1.15	1.03	22:1n-7	0.22	0.44
16:3n-4	0.78	0.85	22:2n-6	1.00	1.00
17:1	2.35	1.99	21:5n-3	1.41	1.28
16:3n-1	0.71	0.86	22:4n-6	1.00	1.01
16:4n-1	0.68	0.78	22:5n-6	0.90	0.92
18:0	0.81	0.75	22:4n-3	2.07	1.71
18:1n-13	0.85	0.86	22:5n-3	4.28	3.21
18:1n-11	12.73	8.83	22:6n-3	1.02	1.01
18:1n-9	3.13	2.47	24:1n-9	0.14	0.20
18:1n-7	1.43	1.30			

Table 5.4: Dietary fatty acid set and four potentially informative extended dietary fatty acids.

Dietary FA	Extended Dietary FA
16:2n-6	14:0 (A)
16:2n-4	18:0 (B)
16:3n-6	18:1n-7 (C)
16:3n-4	18:1n-9 (D)
16:4n-1	
18:2n-6	
18:2n-4	
18:3n-6	
18:3n-4	
18:3n-3	
18:3n-1	
18:4n-3	
18:4n-1	
20:1n-11	
20:1n-9	
20:1n-7	
20:2n-6	
20:3n-6	
20:4n-6	
20:3n-3	
20:4n-3	
20:5n-3	
22:1n-11	
22:1n-9	
22:1n-7	
21:5n-3	
22:4n-6	
22:5n-6	
22:4n-3	
22:6n-3	

measuring how well the variability in the seal FA signatures was explained by the predicted prey FA signatures (C. Stewart, unpublished), and 3) correspondence of final experimental diet estimate to the expected range. For this last criteria, if the experimental diet estimate fell within the range established by its expected signatures, it was scored as zero. If it fell outside this range, the absolute value of the distance above or below the range was recorded. These values were then summed to give an experiment-wide score for each set of modelling parameters.

Once a single set of model parameters was decided upon, diet estimates and standard errors were then generated. These estimates were averaged across each experiment and a standard error of the average was calculated according to the formula: $SE = [(1/n \ se_i^2 + 1/(n-1) \ (est_i - est_{avg})^2)/n]$ which takes into account the variability within a seal (first term) and the variance among seals (second term).

Results

Due to the preliminary nature of the Spring 1998 experiment (i.e., only 4 seals fed for a short period, as well as poor response to diets fed) and the severe health abnormalities caused by the experimental diet in the Spring 1999 experiments (See Chapter 4), results from these experiments are not presented. I thus focus solely on the remaining three experiments (Fall 1999, Spring 2000A and Spring 2000B) and hereafter "all" refers to those experiments only.

In all experiments the FA composition of the blubber samples taken from the right and left flanks of the animals were similar (Figures 5.1-5.3). This was true both for

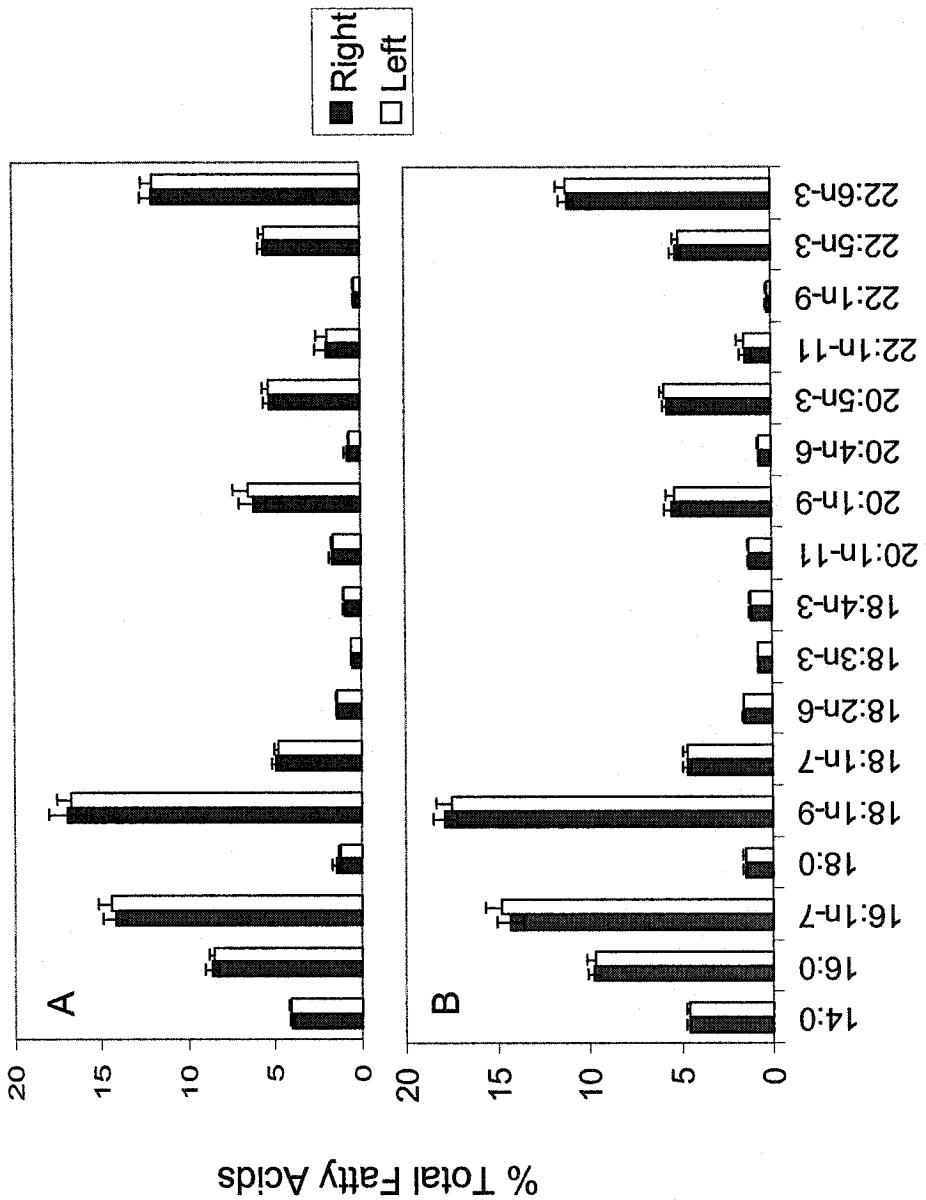

Figure 5.1: Selected fatty acids in whole blubber samples taken from the right and left flank of seals at A) the beginning and B) the end of the Fall 1999 experimental period (n = 8). Values represent mean ± SE.

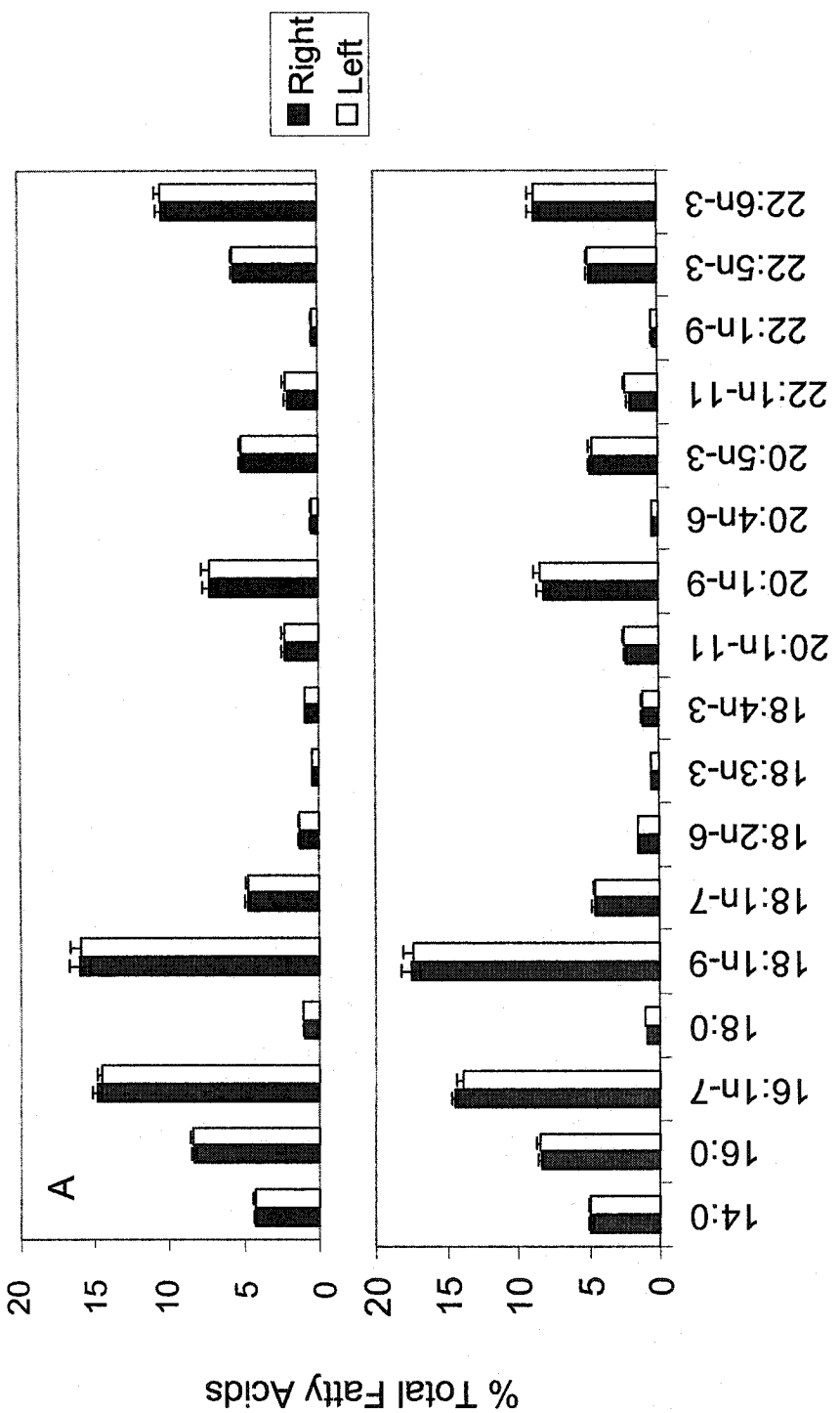

Figure 5.2: Selected fatty acids in whole blubber samples taken from the right and left flank of seals at A) the beginning and B) the end of the Spring 2000A experimental period (n = 10). Values represent mean ± SE.

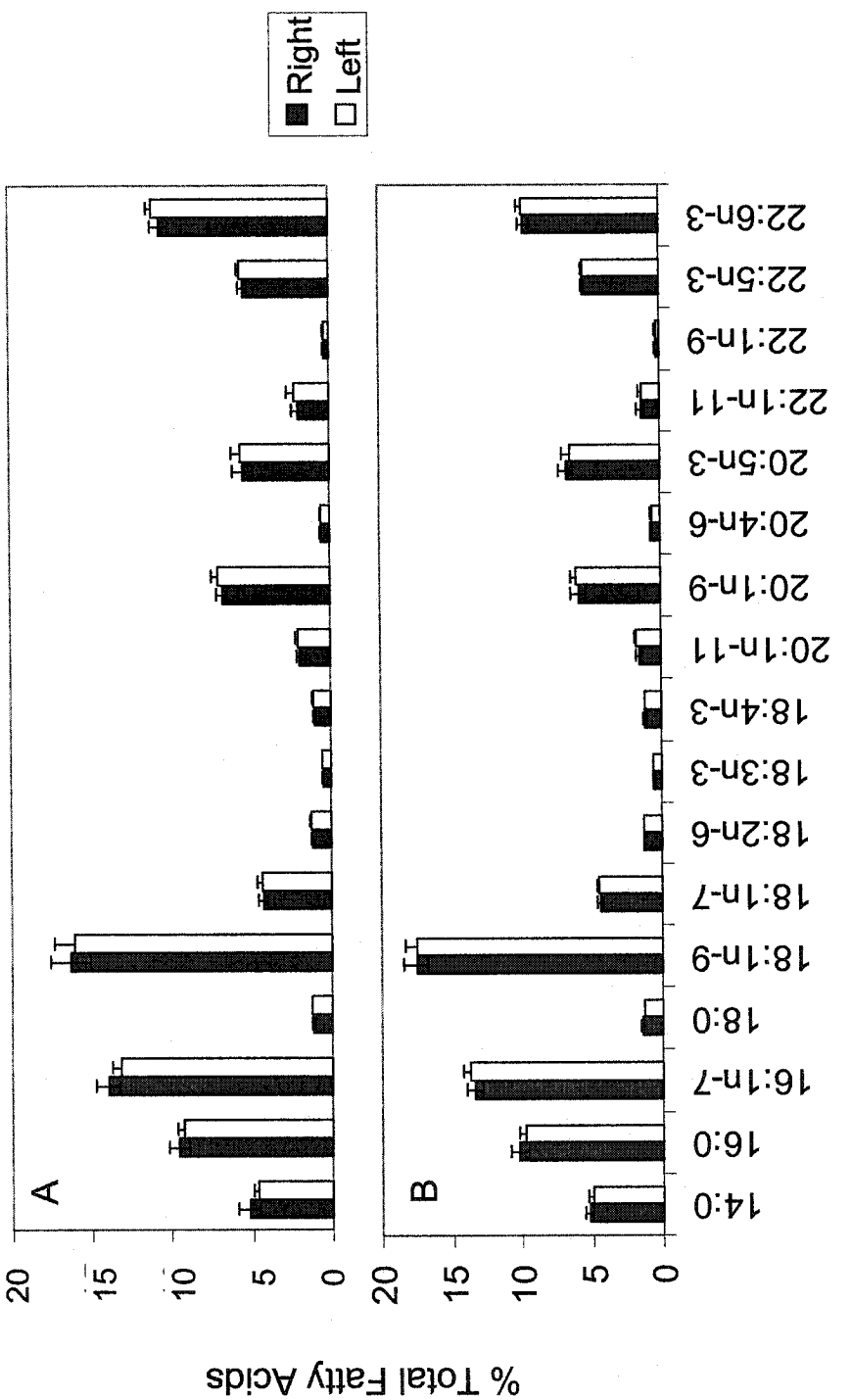

Figure 5.3: Selected fatty acids in whole blubber samples taken from the right and left flank of seals at A) the beginning and B) the end of the Spring 2000B experimental period (n = 10). Values represent mean ± SE.

samples taken at the beginning and at the end of the experiments, indicating that the incorporation of dietary FA both before and during the experimental diet trial was symmetrical in the flank region of the animals.

At the beginning of the experiments, the inner blubber layer contained a greater percentage of SFA (17.5 ± 0.61% vs. 14.7 ± 0.20%, $p < 0.01$) while the outer layer contained more short-chain (< 18 carbons) MUFA (SC-MUFA; 19.7 ± 0.36% vs. 14.3 ± 0.36%, $p < 0.01$) (Figure 5.4). The inner layer also had a slightly greater proportion of PUFA (31.9 ± 0.71% vs. 28.8 ± 0.49%, $p < 0.01$), while the contribution to each layer by the long-chain MUFA (LC-MUFA, ≥ 18 carbons) was similar (36.5 ± 0.86% vs. 36.7 ± 0.59%, inner and outer layers respectively, $p = 0.81$). These patterns did not change over the course of the experiments (data not shown). The dominant SFA were 14:0 and 16:0 (Table 5.5). The major FA in the SC-MUFA class were 14:1n-5 and 16:1n-7. Many FA made a significant contribution to the LC-MUFA class, including the 18:1 isomers, 20:1n-11, 20:1n-9 and 22:1n-11. Two of these LC-MUFA had greater concentrations in the inner layer (20:1n-9 and 22:1n-11), while the rest had greater concentrations in the outer layer. The major PUFA in these animals were 20:5n-3, 22:5n-3 and 22:6n-3.

The concentrations of FA varied considerably among the three experimental diets (Figures 5.5-5.7, Appendix 5.1). The concentrations of the long-chain MUFA 20:1n-9 and 22:1n-11 were very high in the diet of Spring 2000A, moderate in that of Fall 1999 and quite low in the diet of Spring 2000B. On the other hand, the diet of Spring 2000B contained almost twice as much 20:5n-3 than did the diet of Spring 2000A. In all experiments, there was an overall tendency for the FA composition of the blubber to be more similar to the diet signature at the end of the experiment compared to the beginning.

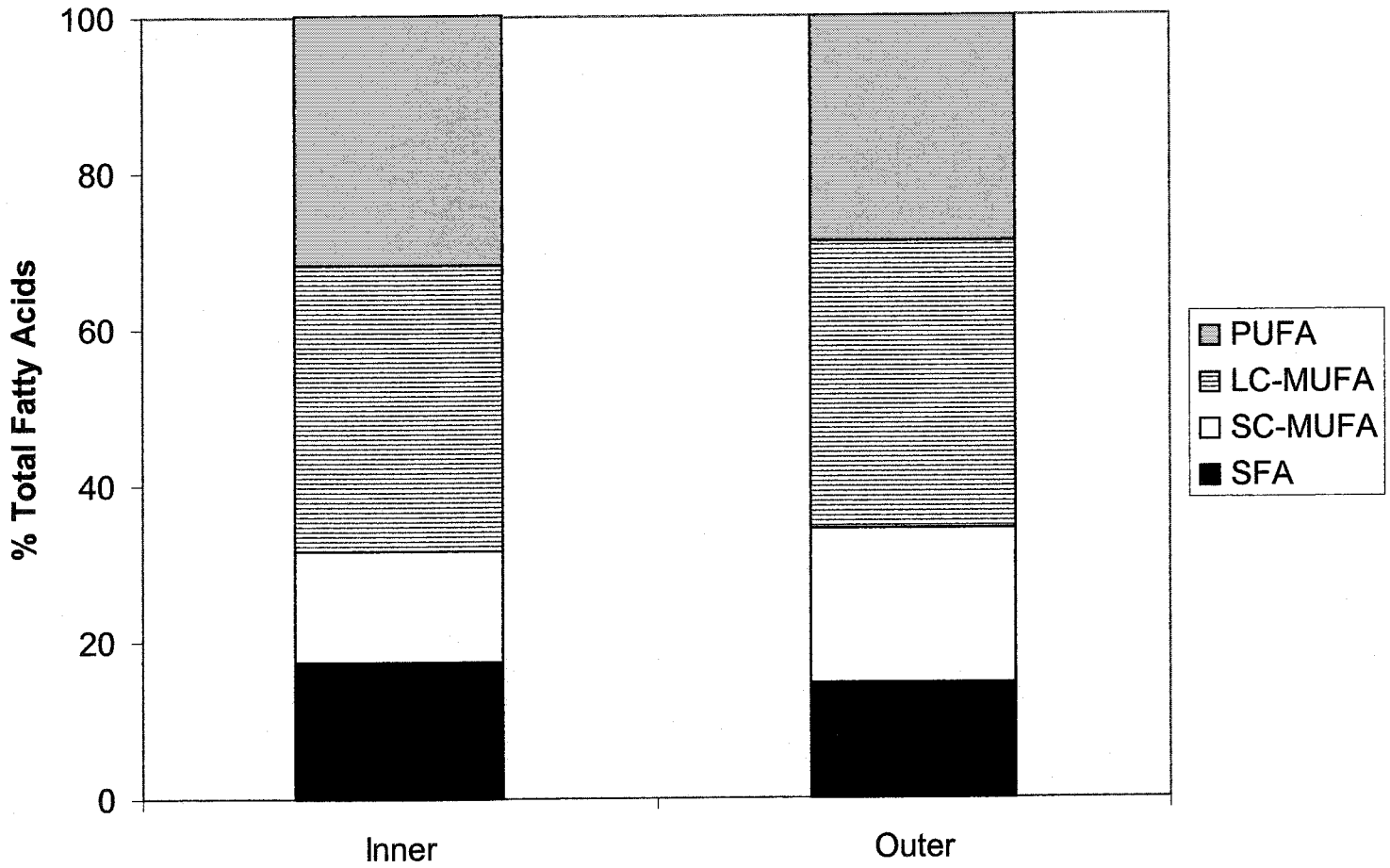

Figure 5.4: Fatty acid classes in the inner and outer blubber samples taken at the beginning of experimental periods (n = 28). SFA = saturated, SC-MUFA = short chain monounsaturated, LC-MUFA = long chain monounsaturated, PUFA = polyunsaturated

Table 5.5: Fatty acid composition of inner and outer blubber layers sampled prior to the beginning of the Fall 1999, Spring 2000A and Spring 2000B experiments. Values represent mean ± SE.

Fatty Acid	Intial Inner n = 28	Initial Outer n = 28
14:0	4.9 ± 0.33	4.1 ± 0.06
14:1n-5	0.8 ± 0.03	1.2 ± 0.03
15:0	0.3 ± 0.01	0.3 ± 0.00
16:0	9.5 ± 0.33	8.4 ± 0.13
16:1n-11	0.4 ± 0.01	0.6 ± 0.01
16:1n-9	0.4 ± 0.02	0.5 ± 0.01
16:1n-7	12.0 ± 0.36	16.7 ± 0.32
16:2n-4	0.3 ± 0.01	0.3 ± 0.01
16:3n-6	0.5 ± 0.03	0.5 ± 0.01
16:3n-4	0.3 ± 0.02	0.3 ± 0.01
17:0	0.3 ± 0.02	0.3 ± 0.01
16:3n-1	0.0 ± 0.01	0.0 ± 0.01
16:4n-3	0.2 ± 0.01	0.1 ± 0.00
16:4n-1	0.5 ± 0.05	0.5 ± 0.03
18:0	1.5 ± 0.10	0.9 ± 0.03
18:1n-11	3.2 ± 0.25	4.4 ± 0.22
18:1n-9	14.9 ± 0.63	16.7 ± 0.41
18:1n-7	4.4 ± 0.16	4.8 ± 0.10
18:1n-5	0.5 ± 0.01	0.5 ± 0.01
18:2n-6	1.3 ± 0.04	1.3 ± 0.03
18:3n-3	0.5 ± 0.02	0.5 ± 0.01
18:4n-3	1.0 ± 0.05	1.0 ± 0.02
20:1n-11	2.0 ± 0.10	1.9 ± 0.08
20:1n-9	7.1 ± 0.37	6.3 ± 0.25
20:1n-7	0.7 ± 0.05	0.5 ± 0.02
20:2n-6	0.2 ± 0.01	0.2 ± 0.01
20:4n-6	0.6 ± 0.06	0.6 ± 0.02
20:4n-3	0.5 ± 0.02	0.5 ± 0.01
20:5n-3	5.6 ± 0.29	5.5 ± 0.17
22:1n-11	3.0 ± 0.33	1.2 ± 0.14
22:1n-9	0.5 ± 0.04	0.3 ± 0.02
21:5n-3	0.4 ± 0.01	0.4 ± 0.01
22:5n-6	0.2 ± 0.01	0.2 ± 0.01
22:5n-3	6.2 ± 0.16	5.1 ± 0.08
22:6n-3	12.0 ± 0.33	10.5 ± 0.28

In Fall 1999, the final blubber FA composition was significantly closer to the diet FA composition than was the initial blubber FA composition ($P = 0.012$, paired-t tests on K-L distances). When only the inner, metabolically more active, blubber layer was considered the correspondence was even closer ($P = 0.006$). In Spring 2000A, for either whole blubber or inner blubber compositions, the same trend toward the experimental diet was observed, although these trends were not statistically significant ($P = 0.075$ and $P = 0.068$, respectively). In Spring 2000B, the final blubber FA composition was again significantly closer to the diet FA composition than was the initial blubber FA composition ($P = 0.002$) using either the whole or inner blubber composition.

Although these overall trends were evident, not all FA concentrations changed in the expected direction of the diet (Figures 5.5-5.7). For example, the concentration of 18:1n-9 was greater at the end of the feeding period than the beginning despite the fact that the concentration in the diet was lower than in the initial blubber samples. The FA, 16:1n-7, also remained high in the blubber despite much lower levels in the diet. In the Fall 1999 and Spring 2000A experiments, the concentration of 22:1n-11 in the blubber did not change in the direction or degree expected based on the level of this FA in the experimental diets (Figures 5.5 and 5.6). Finally, 22:6n-3 appeared to be responsive to changes in diet while 20:5n-3 and 22:5n-3 were less so (Figures 5.5-5.7). These results suggest that the levels of individual FA in the blubber were strongly influenced by metabolism.

When the initial blubber FA compositions were modelled, any predicted contribution by the experimental diets were clearly false positive identifications. I tested two sets of calibration coefficients (CC) and 16 FA sets (Tables 5.3 and 5.4). In the Fall

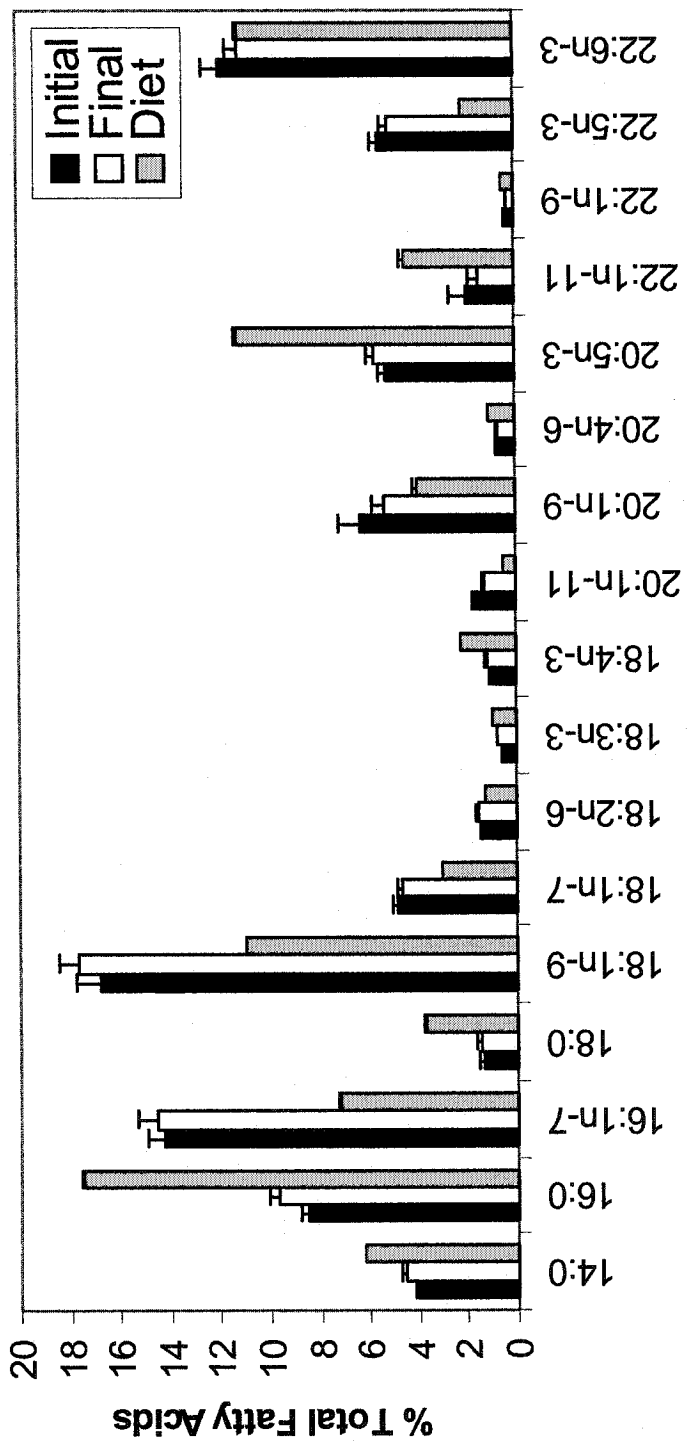

Figure 5.5: Initial and final proportion of selected fatty acids in whole blubber samples in comparison with that of the experimental diet for the Fall 1999 experiment (n = 8). Values represent mean ± SE.

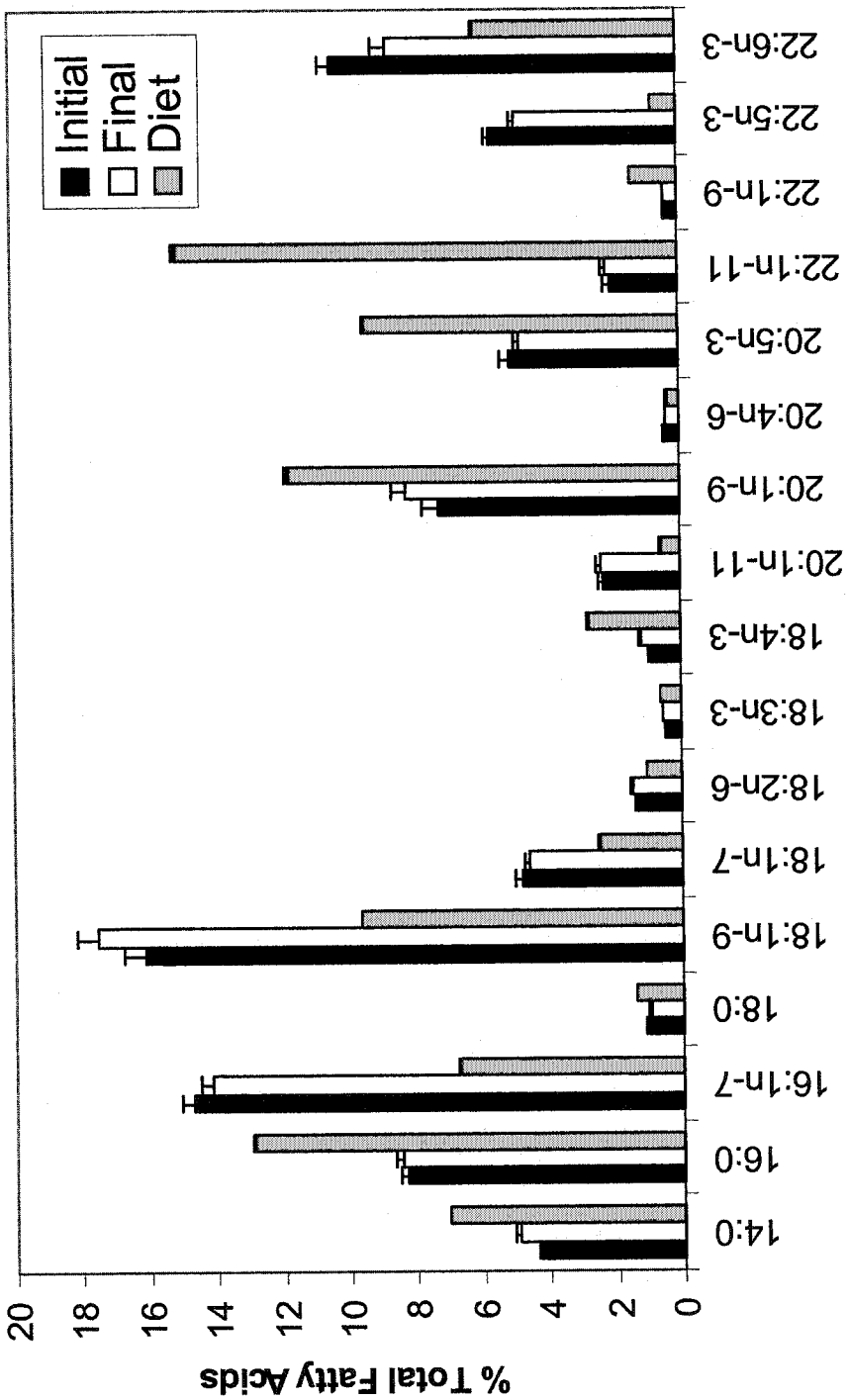

Figure 5.6: Initial and final proportion of selected fatty acids in the whole blubber samples in comparison with that of the experimental diet for the Spring 2000A experiment (n = 10). Values represent mean ± SE.

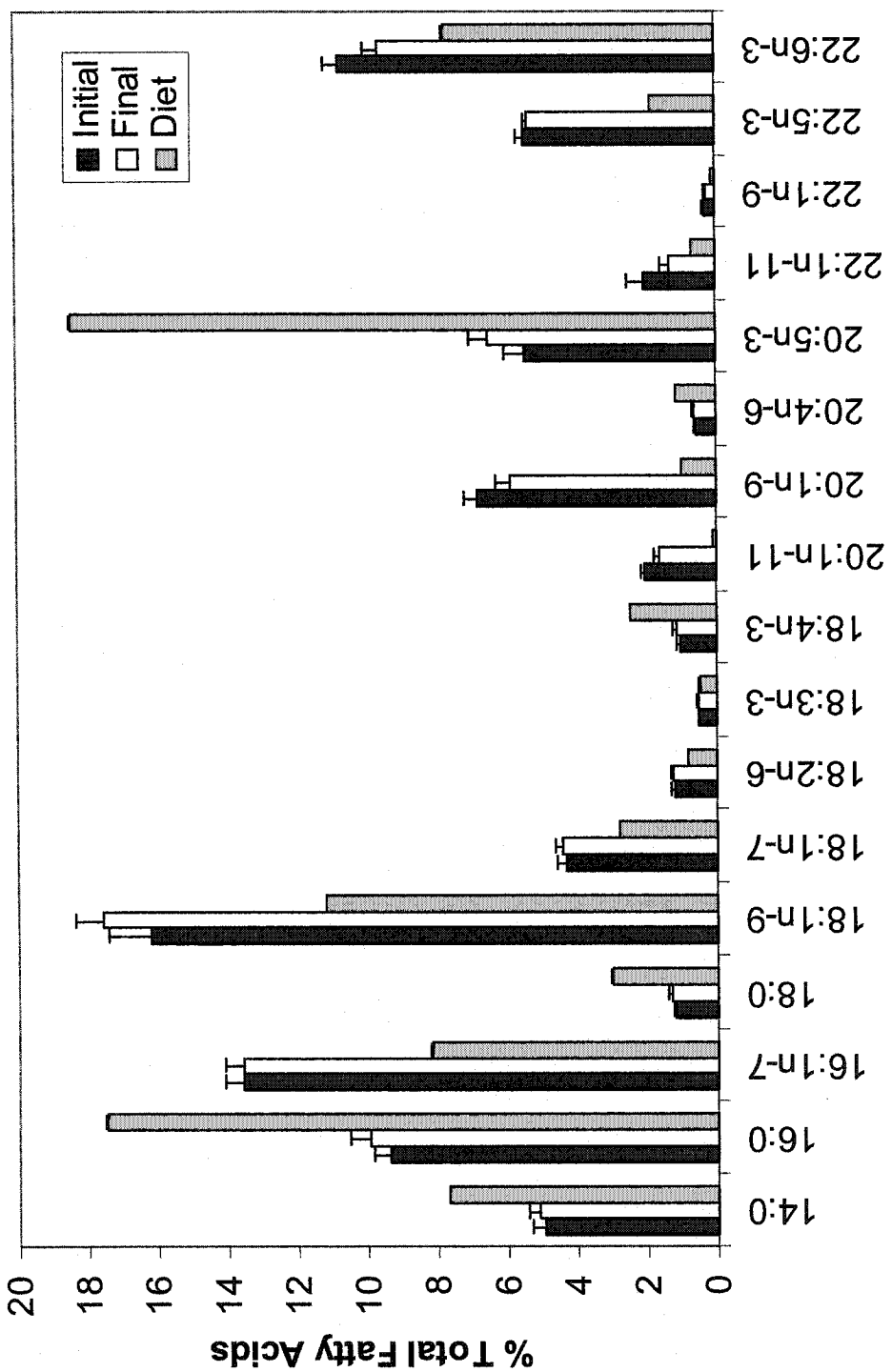

Figure 5.7: Initial and final proportion of selected fatty acids in the whole blubber samples in comparison with that of the experimental diet for the Spring 2000B experiment (n = 10). Values represent mean ± SE.

1999 and Spring 2000A experiments (Figures 5.8 and 5.9, respectively), it was not clear whether the Grey.Harp CC or the Grey.Harp.Pup CC performed better. In Fall 1999, the former tended to produce lower false positives, but in the Spring 2000A experiment the opposite was true. What is clear from these data is that the FA set used strongly affected the level of false positive identification. FA sets dietary+D, dietary+CD, and dietary+BCD (Table 5.4) performed well with both the Fall 1999 and Spring 2000A experiments (Figures 5.8 and 5.9). Furthermore, the inner blubber FA samples performed better, resulting in lower levels of false positive identifications (Figures 5.8A vs. 5.8B, and 5.9A vs. 5.9B).

Both initial and final blubber samples were then modeled using the same two CC sets and 16 FA subsets and the goodness of fit was calculated for each combination. In all cases the goodness of fit of the diet estimates were typically high (> 0.8), but varied in consistent patterns (Figures 5.10-5.11). The FA set dietary+D produced as high or higher goodness of fit values than the other two FA sets identified above as reducing false positives in initial blubbers. The results from the goodness of fit tests also indicated that, in general, the Grey.Harp CC produced estimates with slightly to markedly higher goodness of fit values.

The final criteria upon which the various model parameters were tested was how well the final estimates of the experimental diets' contributions to the overall diet fit within the range generated by modelling the expected final signatures calculated from the possible deposition scenarios (see Methods). When this was tested, again, the Grey.Harp CC clearly performed as well or better than the Grey.Harp.Pup CC (Figures 5.12 and 5.13). Once again, dietary+D performed as well or better than the other two potential FA

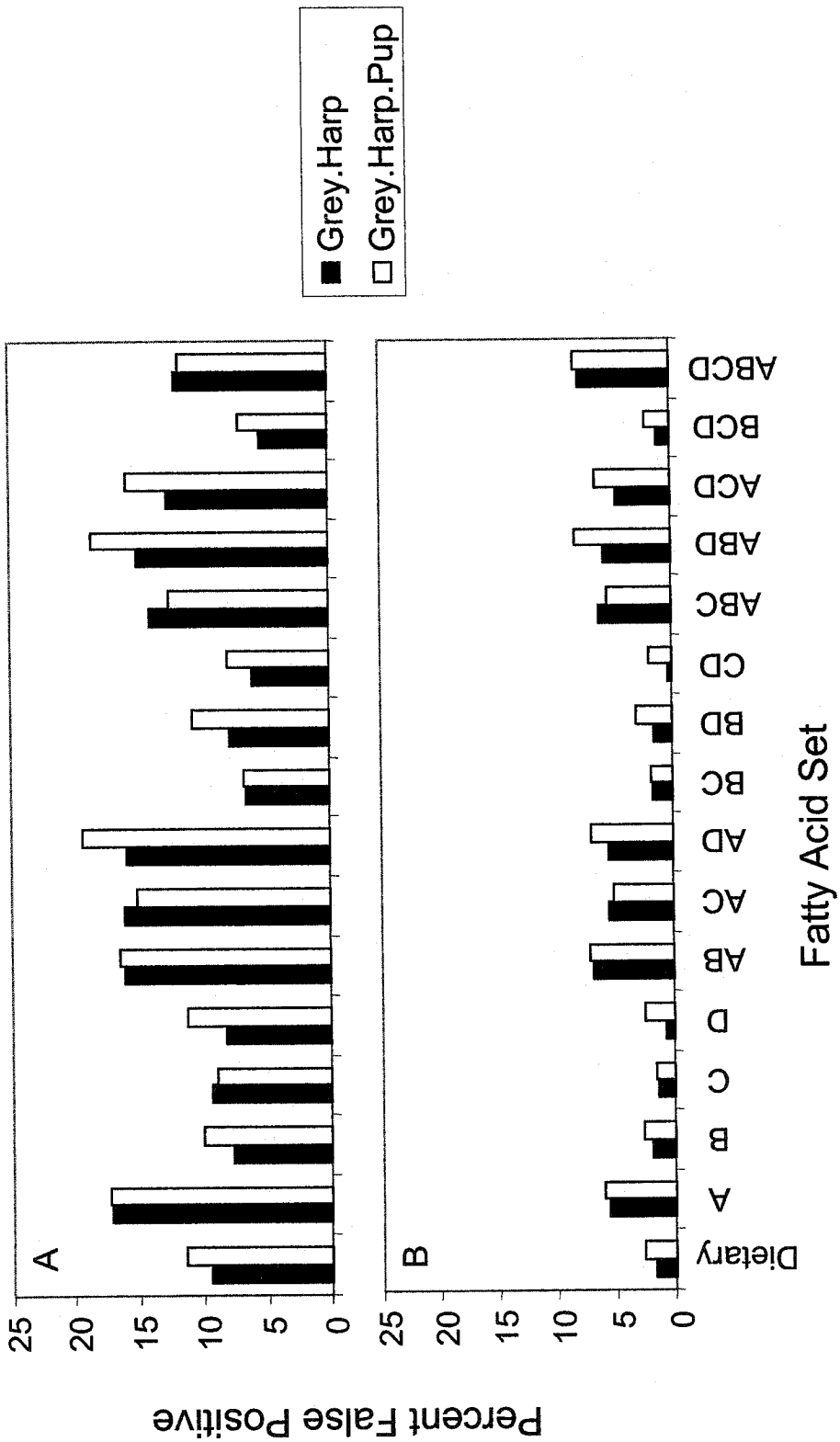

Figure 5.8: Percent false positive identifications made by the QFASA model when initial A) whole and B) inner blubber samples from the Fall 1999 experiment were modelled using either the Grey.Harp or Grey.Harp.Pup calibration coefficients and one of the 16 fatty acid sets being tested (n = 8).

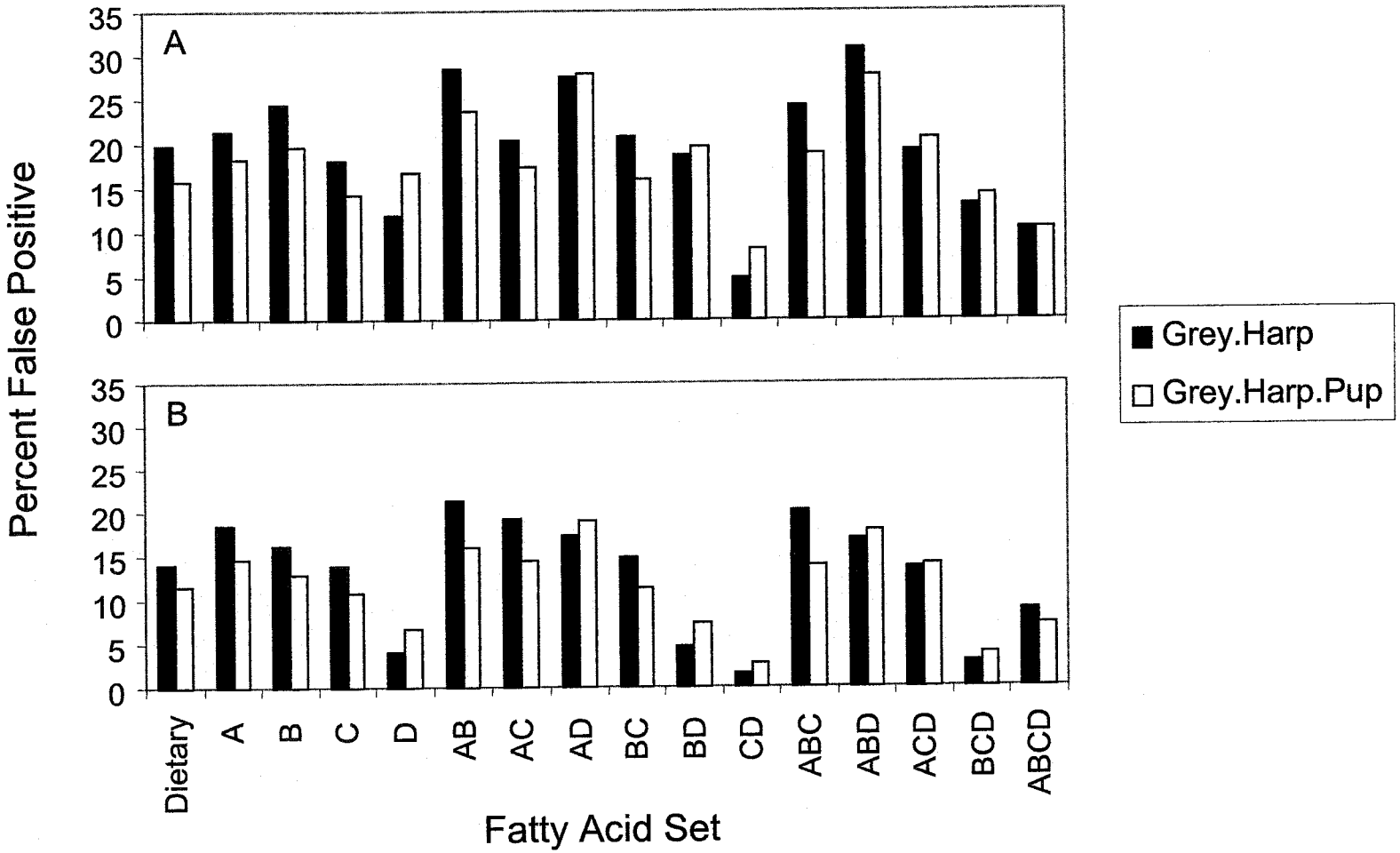

Figure 5.9: Percent false positive identifications made by the QFASA model when initial A) whole and B) inner blubber samples from the Spring 2000A experiment were modelled using either the Grey.Harp or Grey.Harp.Pup calibration coefficients and one of the 16 fatty acid sets being tested (n = 10).

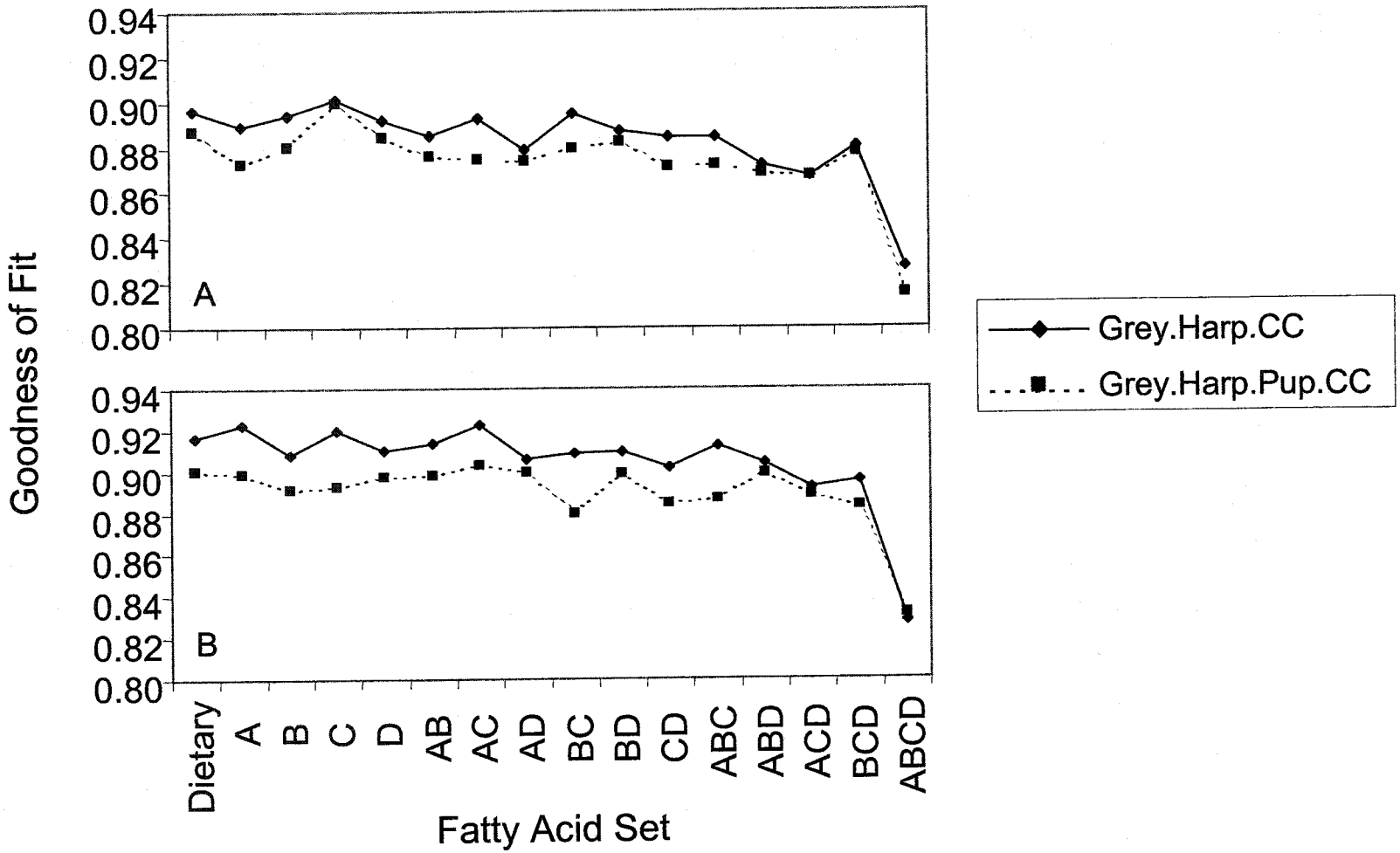

Figure 5.10: Measure of the goodness of fit of diet predictions made by the QFASA model based on whole blubber samples from A) the beginning and B) the end of the Fall 1999 experiment modelled using either the Grey.Harp or Grey.Harp.Pup calibration coefficients and one of the 16 fatty acid sets being tested (n = 8).

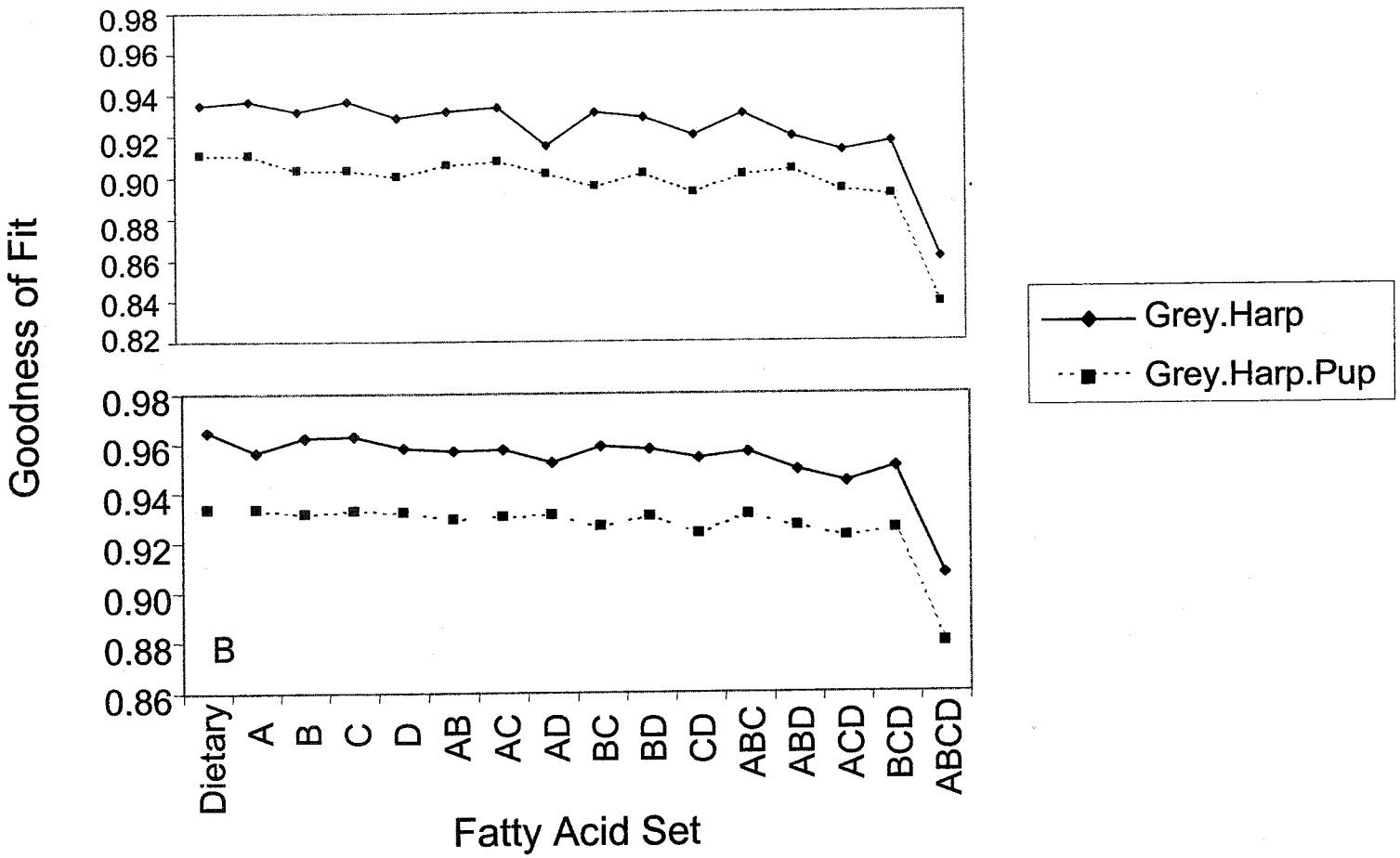

Figure 5.11: Measure of the goodness of fit of diet predictions made by the QFASA model based on whole blubber samples from A) the beginning and B) the end of the Spring 2000A experiment modelled using either the Grey.Harp or Grey.Harp.Pup calibration coefficients and one of the 16 fatty acid sets being tested (n = 10).

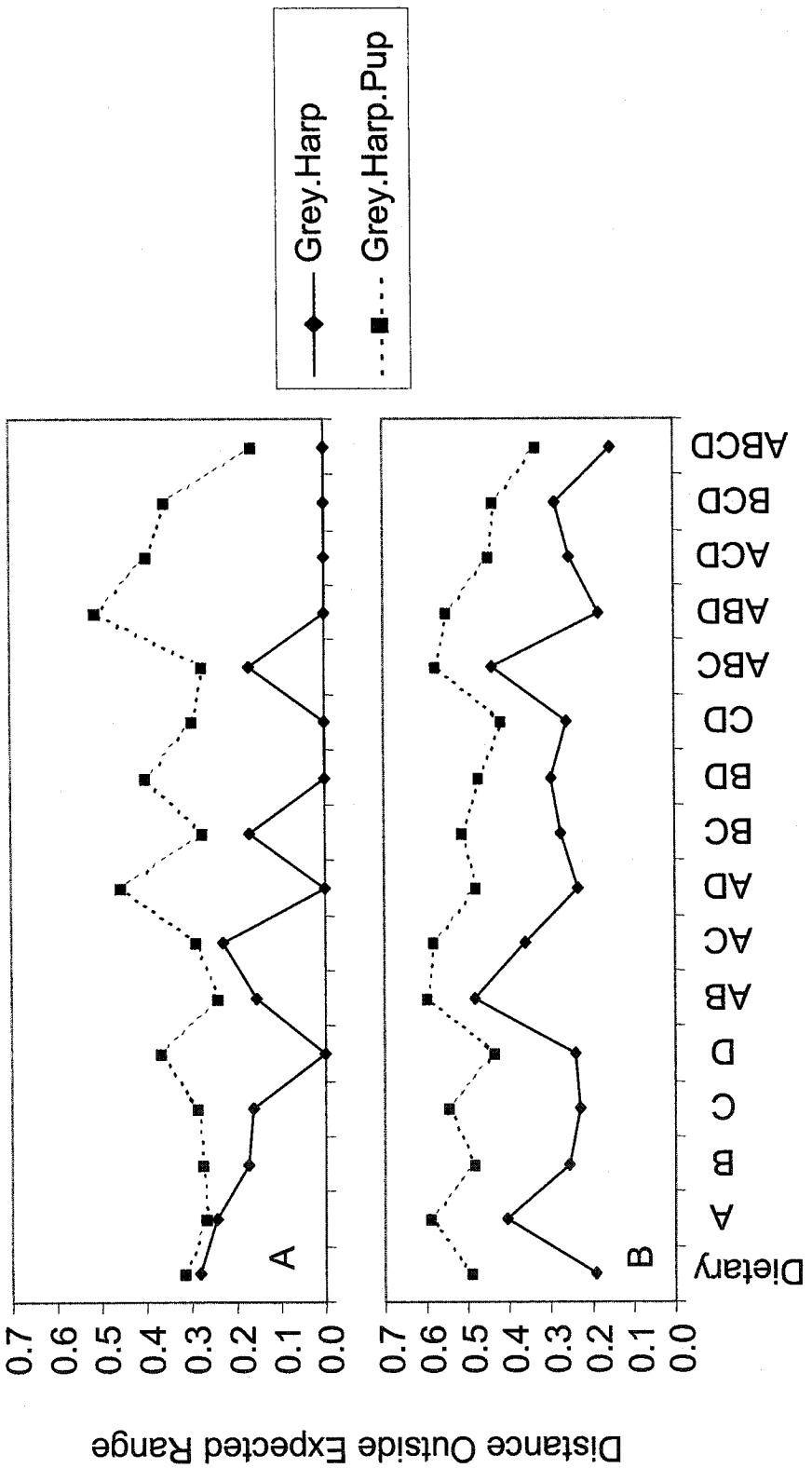

Figure 5.12: Measure of the distance outside the expected range of model estimates of the contribution of the experimental diet to the overall diet at the end of the experimental period based on (A) whole and (B) inner blubber samples from the Fall 1999 study animals (n = 8).

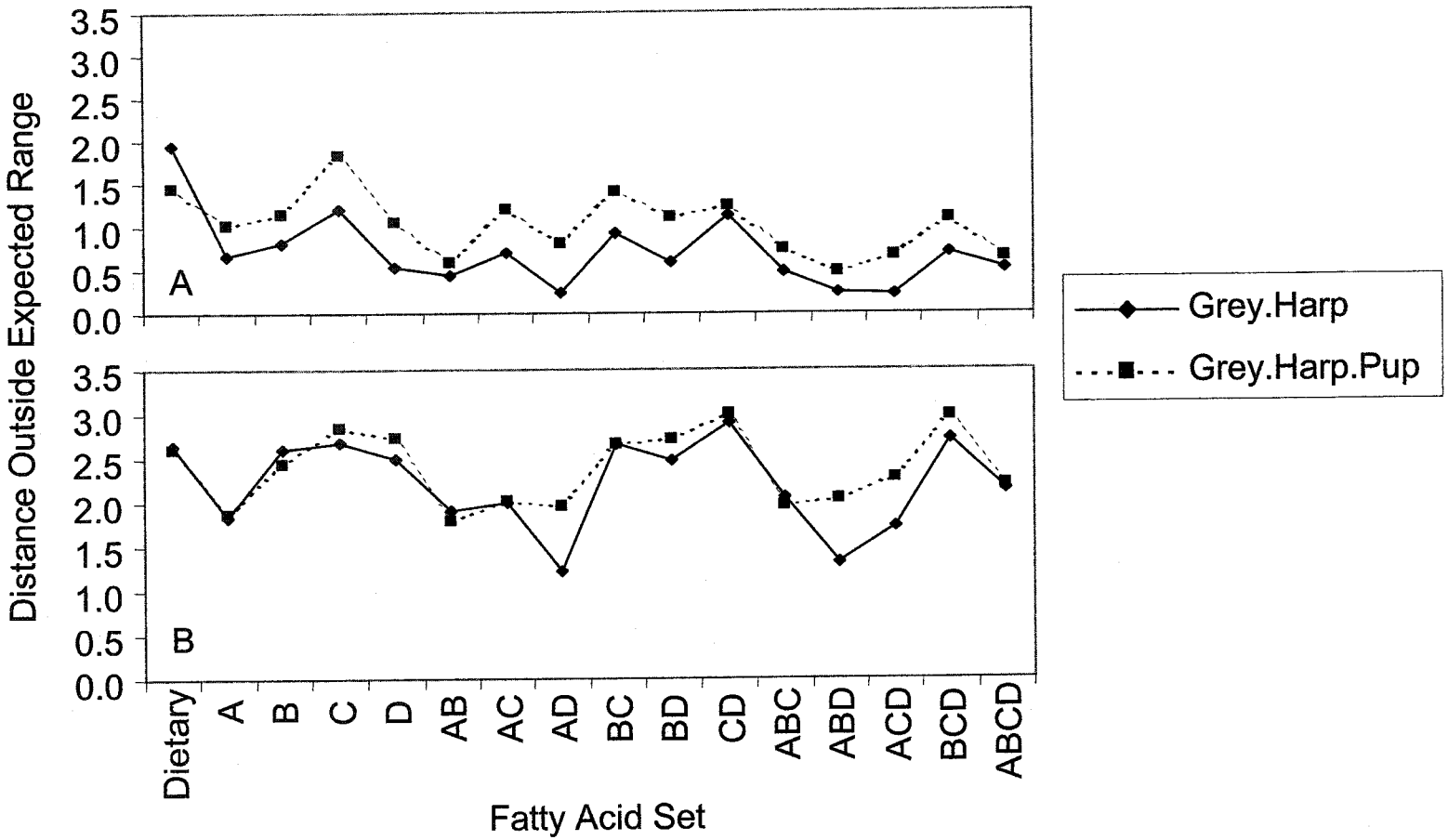

Figure 5.13: Measure of the distance outside the expected range of model estimates of the contribution of the experimental diet to the overall diet at the end of the experimental period based on (A) whole and (B) inner blubber samples from the Spring 2000A study animals (n = 10).

sets.

Based on the above analyses, the Grey.Harp CC and FA set dietary+D (Tables 5.3 and 5.4) were chosen as the parameters to be used in the final modelling of all data. As anticipated, in all three experiments the experimental diet was estimated to contribute a significantly greater proportion to the diet modelled at the end of the experiment than at the beginning (Figures 5.14-5.16, see also Appendix 5.2 for full results). The estimated contribution to diet of the experimental diet at the end of the experiments was also greater when the inner blubber layer was modeled than when the whole blubber layer was modeled (Figures 5.14A vs. 5.14B, 5.15A vs. 5.15B, 5.16A vs. 5.16B). Furthermore, the experimental diet's estimated contribution to the overall diet at the end of the experiments was significantly greater than zero in all cases. These results demonstrate that after only a two-week feeding period the experimental diets made a significant contribution to lipids stored in the blubber of the experimentally fed seals.

In Fall 1999, the major species identified as contributing to the diet prior to the experimental period included northern sandlance, redfish and winter skate (Figure 5.14 A and B). In Spring 2000A, northern sandlance and redfish were again the primary species identified, with winter skate making a smaller contribution (Figure 5.15 A and B). Northern sandlance, redfish and winter skate were again estimated to be the primary contributors prior to the Spring 2000B experiment (Figure 5.16 A and B). Although cod, herring, and the flounders appeared in several of the model outputs, the large standard errors associated with the estimates suggest that in most cases the true value was close to zero. Generally speaking, the species identified as contributing to diet at the end of the

experimental period did not differ from those identified prior to feeding, but their contribution to the diet varied.

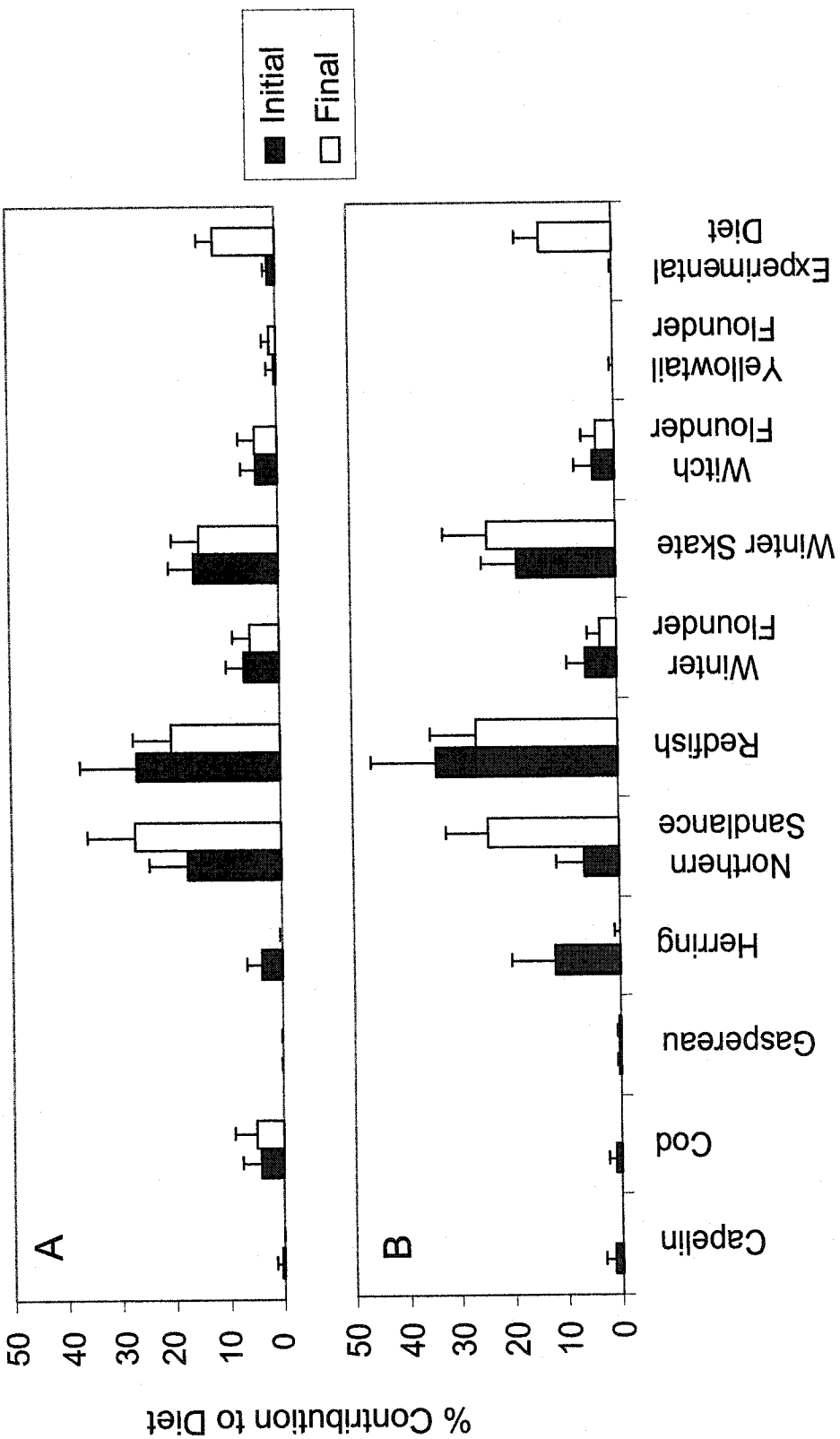

Figure 5.14: Model estimates of the contribution of prey species to diet (mean ± SE) based on (A) whole and (B) inner blubber samples from Fall 1999 study animals.

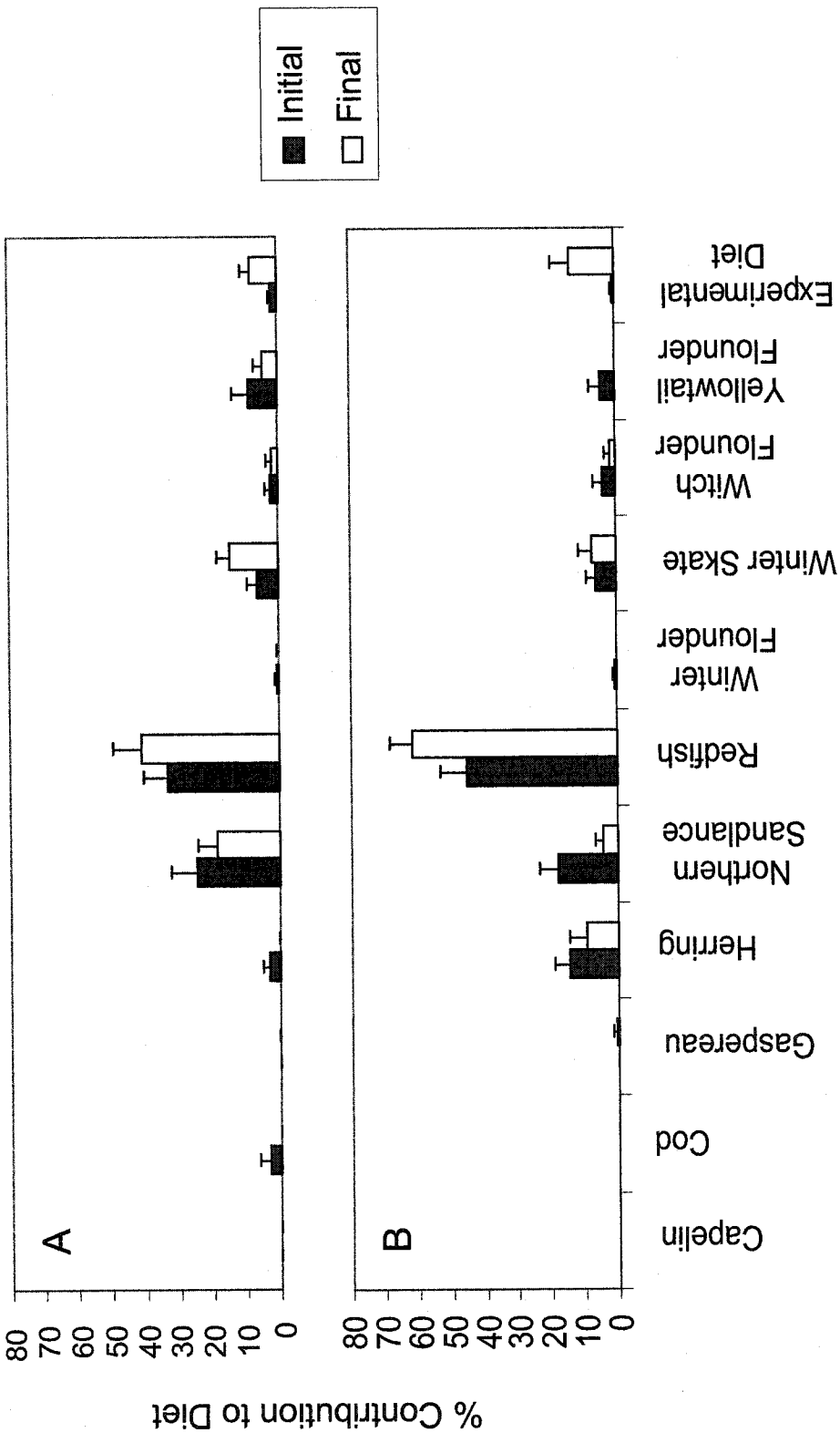

Figure 5.15: Model estimates of the contribution of prey species to diet (mean ± SE) based on (A) whole and (B) inner blubber samples from Spring 2000A study animals.

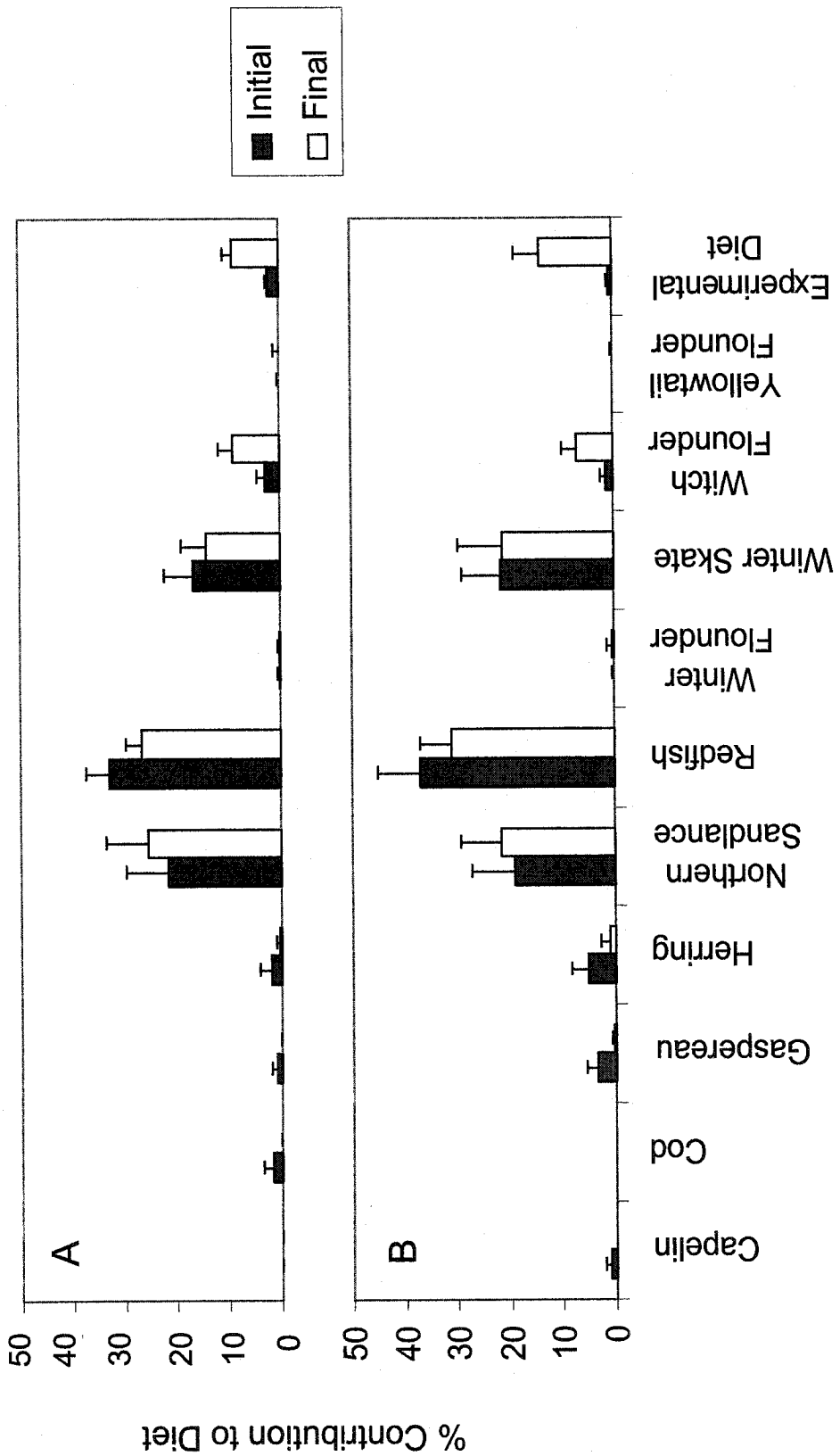

Figure 5.16: Model estimates of the contribution of prey species to diet (mean ± SE) based on (A) whole and (B) inner blubber samples from Spring 2000B study animals.

Discussion

FA Stratification and Heterogeneity in Blubber

It is well known that cetaceans exhibit heterogeneity in FA composition of the blubber across the body surface and vertical stratification of FA composition across the blubber depth, both of which are likely related to site-specific functions of adipose tissue (Ackman *et al.* 1965, 1975a, 1975b, Lockyear *et al.* 1984, Koopman *et al.* 1996, Hooker *et al.* 2001, Koopman *et al.* 2002, 2003). Few studies, however, have looked at these properties in pinniped blubber (Käkelä *et al.* 1993, Käkelä and Hyvärinen 1996, Cooper *et al.* 2001, Best *et al.* 2003). Käkelä and Hyvärinen (1996) showed that differences exist between the FA composition of blubber found on the head, near the flippers and around the girth of ringed seals, but that the FA compositions of samples taken from the dorsal and ventral regions around the girth of the animals were very similar. The data from this study also demonstrate that the blubber FA composition around the girth of the animal is homogeneous at least in comparing samples from the right and left flanks of individuals (Figures 5.1-5.3). This shows that within this region of the animal, the exact site of biopsy sampling does not affect the FA composition determined. The homogeneity of FA composition within this region of the blubber also allows averaging of data from the right and left flanks to calculate a single blubber FA composition. Ackman *et al.* (1975a) suggest that dietary FA are deposited in the ventral region of mysticeti whales more readily than in the dorsal region. If true, this is in contrast to the implications of the data presented here. The symmetrical response of the right and left blubber samples to the

experimental diets indicates a biochemical and functional homogeneity of the blubber in this region of grey seals (Figures 5.1b, 5.2b, and 5.3b).

In contrast to the homogeneity across the girth, vertical stratification of the blubber FA composition of girth samples has been shown previously in pinnipeds (ringed seals, Käkelä and Hyvärinen (1996); grey seals, Cooper *et al.* (2001); southern elephant seals (*Mirounga leonine*), Best *et al.* (2003)). Again, my current results were consistent with these earlier findings (Figure 5.4, Table 5.5). In both pinnipeds and cetaceans there is a tendency for higher levels of SFA, certain MUFA (e.g. 20:1n-9 and 22:1n-11) and PUFA (e.g. 20:5n-3, 22:5n-3 and 22:6n-3) in the inner blubber layer and higher levels of MUFA that are readily synthesized endogenously in the outer layer (Käkelä and Hyvärinen 1996, Koopman *et al.* 1996, Hooker *et al.* 2001, Best *et al.* 2003, Olsen and Grahl-Nielsen 2003, Table 5.5). It is generally accepted that the inner blubber layer is more metabolically active (Ackman *et al.* 1975a, Lockyer *et al.* 1984, Koopman *et al.* 1996, Hooker *et al.* 2001, Iverson 2002). Studies using isotopically labelled dietary FA indicate that although dietary FA have access to the outer blubber layer, they have greater access to the inner layer (Budge *et al.* 2004, M. Cooper unpublished data). The higher levels of Δ9 MUFA (e.g. 14:1n-5, 16:1n-7, 18:1n-9) in the outer layer and SFA in the inner layer imply either a greater activity of the Δ9 desaturase in the outer layer or a poor mobilization of these MUFA such that they build up in the older, outer layer FA (Koopman *et al.* 1996). Clearly, vertical stratification of FA composition affects the use of blubber FA to estimate diet. The FA signature and, thus, the dietary estimate depend on what portion of the blubber layer is analysed. Because the inner blubber layer is the more metabolically active, diet estimates made using only the inner FA signature

represent more recent consumption than those made using the outer or full blubber FA signatures (Cooper *et al.* 2001).

Dietary Effects on Blubber FA Composition

Several studies have shown that when a diet of known FA composition is consumed, the adipose tissue FA signature tends to shift in the direction of the diet (Iverson *et al.* 1995, Kirsch 1997, Kirsch *et al.* 1998, 2000). In the latter three studies, the FA composition of the diet consumed prior to the experimental period was known. In the case of Iverson *et al.* (1995) the study animals were new-born hooded seal pups and obviously had not been feeding prior to consumption of their mothers' milk. This allowed for the comparison of the levels of individual FA between the control and experimental diets as well as between the initial and final blubber FA signatures, such that one could clearly correlate a shift in the level of a FA in the blubber signature to a corresponding shift in the level of that FA in the diet. My results are consistent with these previous findings in so far as the final blubber signatures were more similar to the diet than were the initial blubber signatures (Figures 5.5-5.7). A caveat, however, is required for interpreting my data. Because my study animals were wild seals, the control diet (that consumed prior to the experimental period) was not known. This means that while a greater similarity between the level of a FA in the final blubber signatures and the experimental diet signature implies a shift in the direction of the diet, this cannot be said with certainty.

The concentration of individual FA whose levels did not shift in the direction of the experimental diet are likely influenced by metabolism within the seals. For example,

the concentrations of the MUFA 16:1n-7 and 18:1n-9 in the blubber remained high, or increased, despite much lower levels in the experimental diet (Figures 5.5-5.7). Both 16:1n-7 and 18:1n-9 are readily synthesized through Δ9-desaturation of endogenous 16:0 and 18:0, respectively (Cook 1991). The concentration of these SFA in the blubber also appeared to be rather resistant to change (Figures 5.5-5.7), implying that dietary 16:0 and 18:0 can also enter this desaturation pathway. Results from Budge *et al.* (2004), that showed radioactive 16:1 in the blubber of grey seals after ingestion of ^{3}H labelled 16:0, support this conclusion.

Although 20:1n-9 concentration always shifted in the direction of the diet, 22:1n-11 levels remained low or fell despite higher concentrations in the diet than the initial blubber samples (Figures 5.5-5.7). Both these long-chain MUFA, when found in the predator, are primarily exogenous in origin and can be good indicators of diet (Lee *et al.* 1971, Ackman 1980, Ackman *et al.* 1980, Falk-Petersen *et al.* 1990, Iverson 1993, Budge *et al.* 2002, Iverson *et al.* 2002). These FA are under-represented in adipose tissue relative to the diet, with 22:1n-11 being more so than 20:1n-9 (Holland *et al.* 1990, Lin and Connor 1990, Lin *et al.* 1993, Kirsch *et al.* 1998, Kirsch *et al.* 2000, Cooper *et al.* 2001, Iverson *et al.* 2004). This is likely a result of 22:1 isomers being better substrates for peroxisomal β-oxidation than 20:1 isomers (Bremer and Norum 1982). Ultimately, this difference in enzyme affinity for the two substrates causes 20:1n-9 to reflect diet more directly than does 22:1n-11.

As marine carnivores, seals are exposed to potential prey items that are high in 20:5n-3 and 22:6n-3 (Ackman *et al.* 1980, Iverson *et al.* 1997b, Budge *et al.* 2002, Iverson *et al.* 2002). The metabolism of these long-chain FA is, therefore, of importance

in studying the diets of these animals. Studies have indicated that the relative accumulation of 20:5n-3, and to a lesser extent 22:6n-3, in adipose tissue, muscle and liver, is low in species such as humans, rats and rabbits (Sinclair and Gale 1987, Wood *et al.* 1987, Lin and Connor 1990, Jandacek *et al.* 1991, Sheppard and Herzberg 1992, Herzberg and Skinner 1997). Research on rats adapted to fish oil diets has shown that 20:5n-3 is underrepresented relative to 22:6n-3 compared with their content in the diet and that this is due to preferential oxidation of 20:5n-3 (Herzberg *et al.* 1996) as well as its preferential release from muscle, liver and adipose tissue (Sheppard and Herzberg 1992, Herzberg and Skinner 1997). A similar pattern of reduced recovery of 20:5n-3 relative to 22:6n-3 was also found in the blubber of pinnipeds (Iverson *et al.* 2004). However, animals with an abundance of these n-3 PUFA in their diet are likely well-adapted to storing these FA in adipose tissue. Iverson *et al.* (1995) showed that the levels of 20:5n-3 and 22:6n-3 in hooded seal pups just prior to weaning were essentially identical to the levels in their milk diet. Kirsch *et al.* (2000) also showed that the incorporation of 20:5n-3 and 22:6n-3 was predictable, based on the diet, and not reduced relative to other fatty acids.

The processes of inter-conversion between the n-3 FA are important for understanding their levels in blubber and their responses to changes in diet. 20:5n-3 can be elongated to form 22:5n-3 and 22:6n-3 can be retroconverted to form 22:5n-3; 22:5n-3 can then be converted to either 20:5n-3 or 22:6n-3, creating a complete pathway of inter-conversion (Hagve and Christophersen 1986, Grønn *et al.* 1991, Voss *et al.* 1991, Voss *et al.* 1992, Brossard *et al.* 1996). Although each of these reactions are possible, the important determining factor is the extent to which each of these inter-conversions takes

place. In general, 20:5n-3 is readily elongated to 22:5n-3 while 22:5n-3 and 22:6n-3 are more likely to be acylated than they are to be retroconverted such that the physiological end products of n-3 FA modification are the C_{22} FA (Hagve and Christophersen 1986, Grønn *et al.* 1991, Rosenthal *et al.* 1991, Voss *et al.* 1992, Brossard *et al.* 1996). My results correspond well to these patterns of metabolism. Despite the concentration of 20:5n-3 being higher in the experimental diets than the initial blubber samples, the level in the blubber increased only slightly (Figures 5.5, 5.7) or even declined (Figure 5.6). The preferential oxidation of this FA may also play a role in this finding (Herzberg *et al.* 1996). On the other hand, 22:5n-3 concentrations were higher in the final blubber samples than would have been expected based on the very low levels of this FA in the experimental diets (Figures 5.5-5.7). Finally, because 22:6n-3 is more likely to be acylated than retroconverted, it is more directly reflective of diet than are 20:5n-3 and 22:5n-3 (Figures 5.5-5.7).

Optimization of QFASA Model Parameters

Mine is not the first study in which information about individual fat intake and deposition, derived from isotope dilution methods, have been used to estimate the potential effect of the experimental diet on the blubber of study animals (Kirsch *et al.* 2000, Iverson *et al.* 2004). Unique to my calculations, however, is the attempt to account for patterns of FA deposition and mobilization on a physiological time scale. At each meal, the animals consume more nutrients than are required for their immediate needs and deposit FA in the blubber. In the post-prandial period that follows, FA are mobilized for metabolism. Thus, despite being at or below maintenance energy intakes, pinnipeds

still show blubber FA turnover and net deposition (Kirsch *et al.* 2000, Cooper *et al.* 2001). In my study, blubber FA composition was calculated on a daily basis, despite the animals having been fed twice daily, because I didn't feel that the individual fat intake and deposition data were precise enough to warrant the extra level of iteration. The incorporation of calibration coefficients in my calculations further increases their physiological relevance. Regardless of these attempts to mimic the true physiology of the animals, it is important to recognize that these calculations are still based on rather crude assumptions. In all experiments, the estimated final FA compositions were more similar to the actual final signatures than they were to the initial signatures, indicating that my calculations have merit. The finding that the FA compositions estimated based on either 100% or 20% deposition of dietary FA performed equally well implies that the true rate of deposition of dietary FA is intermediate.

FA set used clearly affected the percent false positive identification in the model estimates (Figures 5.8 and 5.9). It was this measure, therefore, that was primarily used to differentiate between the different FA sets tested. The most apparent outcome from these tests was that when 14:0 (A) is included in the FA set, the level of false positive identifications increases. Since little 14:0 is released from the fatty acid synthase enzyme complex (Wakil *et al.* 1983), high tissue concentrations should only be found when it is derived from dietary sources (Nelson 1992). Thus, 14:0 is believed to be a good indicator of diet (Iverson 1993). This reduction in model performance when 14:0 was included was unexpected. The most suitable FA sets appeared to be dietary+D, dietary+CD and dietary+BCD. The common element in these FA sets was the inclusion of 18:1n-9 (D). Although this FA can be readily synthesized by mammals (Cook 1991),

its level in prey species can be quite variable (Budge *et al.* 2002, Iverson *et al.* 2002) potentially explaining its effectiveness when included in the FA set.

The generally lower levels of false positive identifications when inner blubbers were modelled, compared to when whole blubbers were used, may be a result of the vertical stratification of FA composition through the blubber layer. Because the inner blubber layer represents more recent consumption (Cooper *et al.* 2001), there has been less time for the inner FA signature to be affected by modifying processes potentially occurring in the blubber. Thus, the inner FA signature may be more directly reflective of the diet that created it.

The goodness of fit values were all relatively high but were useful in differentiating between the two sets of calibration coefficients tested. The Grey.Harp CC produced generally higher goodness of fit values than did the Grey.Harp.Pup CC (Figures 5.10 and 5.11). The grey seal pups used to derive the Pup calibration coefficients were consuming extremely high fat, milk diets such that the transfer of dietary FA to the blubber was more direct than for the seals consuming a fish diet (Iverson *et al.* 1995, Iverson *et al.* 2004). Thus, the dietary conditions under which the Grey and Harp CC were generated were more similar to my feeding experiments, making it reasonable for the Grey.Harp CC to be more appropriate. When the model estimates generated from the signatures calculated under the assumptions of 100% and 20% deposition of dietary FA were compared to the actual final FA compositions, both calibration coefficient and FA set used appeared to have an impact (Figures 5.12 and 5.13). These results supported the conclusion that the Grey.Harp calibration coefficients were most appropriate for my data set.

While somewhat subjective, an assessment of the latter two criteria were used to differentiate between the three potential FA sets identified based on their performance in the test of false positive identification. In all cases, the FA set dietary+D performed as well or better than dietary+CD or dietary+BCD. This finding was somewhat surprising considering Iverson *et al.* (2004) found that the extended-dietary FA set generally produced better model estimates than the dietary FA set. They suggested that the increased amount of information being incorporated in the model when the extended-dietary FA set was used was responsible for the improved performance. It appears, however, that this may not strictly be the case and that the FA subset used in modelling must be chosen with care.

On a final note regarding model parameters, it appears from my data that the FA subset used has a greater impact on the model estimates than does the set of calibration coefficients (Figures 5.8-5.13). This is really only because the two sets of calibration coefficients that I tested were so similar. Iverson *et al.* (2004) found that the basic use of calibration coefficients and the set chosen for inclusion have a tremendous effect on model estimates, particularly for animals that store their excess fat in blubber as opposed to adipose tissue.

QFASA Estimates of Predator Diet

Although several studies have shown that a change in dietary FA intake can cause the blubber FA composition to shift in the direction of the diet (Iverson *et al.* 1995, Kirsch 1997, Kirsch *et al.* 2000), Iverson *et al.* (2004) were the first to show that this shift in blubber FA composition translates into QFASA model estimates showing appropriate

contributions by the experimental diets. My results corroborate this because, in all three experiments, the QFASA model estimated that the experimental diets made notable contributions to the overall diet at the end of the experiments and that these contributions were significantly greater than zero (Figures 5.14-5.16). This shows that after only a two-week feeding period the experimental diets made a significant contribution to lipids stored in the blubber of the experimentally fed seals. The generally higher goodness of fit values generated when final blubber FA compositions were modelled, compared to initial FA compositions, supports this conclusion (Figure 5.10 and 5.11).

The estimated contribution to overall diet of the experimental diets at the end of the experiments was greater for the inner blubber layer than for the blubber layer as a whole (Figures 5.14A vs. 5.14B, 5.15A vs. 5.15B, 5.16A vs. 5.16B). This implies that there is a greater rate of FA turnover in the inner blubber layer than in the outer. This is consistent with previous notions that the inner blubber layer is more metabolically active (Ackman *et al.* 1975a, Lockyer *et al.* 1984, Koopman *et al.* 1996, Hooker *et al.* 2001, Iverson 2002), and, as such, diet estimates made using only the inner FA signature represent more recent intakes than those made using the outer or full blubber FA signatures (Cooper *et al.* 2001). Because pinnipeds go through periods of fat depletion, associated with fasting, followed by periods of fattening, there is already some insight into the time frame over which blubber FA signatures are integrating the diet. While much longer term experiments than those conducted here will be required to gain a solid understanding of the time scale of FA turnover in these animals, this study shows that the period of consumption represented by the inner blubber layer is most likely longer than two weeks and that the period represented by the blubber as a whole is longer still.

Because the sample sizes in these experiments were quite small (≤ 10), it was not my intent to extrapolate these results to the population as a whole. Nevertheless, some evaluation can be made of the major fish species identified as contributing to the overall diet of my study animals. These included redfish, northern sandlance and winter skate (Figures 5.14-5.16, Appendix 2). Redfish are extremely abundant in the area in which my study animals were likely feeding (DFO 1997, Mahon *et al.* 1998). Redfish are demersal fish normally found in depths between 100-700 m but can also be distributed well up into the water column (DFO 1997). Northern sandlance is abundant in the area around Sable Island and traditional diet studies have shown it to be the most important food item for grey seals foraging in the area (Bowen *et al.* 1993, Bowen and Harrison 1994). It is interesting that winter skate appear so prominently in the diet. Mahon *et al.* (1998) found a close association between northern sandlance and winter skate abundance data from trawl surveys, carried out between Cape Hatteras, North Carolina to Cape Chidley, Labrador from 1970 to 1994, such that the two species were always classified as members of the same demersal fish assemblage. It seems likely that, of the two, northern sandlance was the main target of these grey seals' foraging effort and winter skate was taken out of convenience.

The results of this study demonstrate that even over a relatively brief period of time, FA signatures of a new diet are reflected in predator blubber stores and that as long the differential metabolism of FA within the predator is accounted for, blubber FA can be used to provide an accurate quantitative estimation of diet composition using QFASA. In addition, it appears that while whole blubber provides a longer-term integration of the

dietary history of an individual, modeling the inner half of the blubber layer provides a view of recent diet.

Appendix 1

Fatty acid composition (mean ± SE) of experimental diet and grey seal blubber

Fall 1999:

Fatty Acid	Diet Average n = 12	Blubber Samples			
		Initial n = 8	Final n = 8	Initial Inner n = 8	Final Inner n = 8
14:0	6.2 ± 0.03	4.0 ± 0.13	4.6 ± 0.14	4.2 ± 0.19	5.2 ± 0.18
14:1n-5	0.1 ± 0.00	1.1 ± 0.08	1.0 ± 0.08	0.8 ± 0.09	0.7 ± 0.07
15:0	0.6 ± 0.01	0.3 ± 0.01	0.4 ± 0.01	0.3 ± 0.02	0.4 ± 0.02
16:0	17.5 ± 0.12	8.5 ± 0.33	9.7 ± 0.40	9.2 ± 0.64	11.6 ± 0.67
16:1n-11	0.5 ± 0.01	0.5 ± 0.01	0.6 ± 0.01	0.4 ± 0.02	0.5 ± 0.02
16:1n-9	0.3 ± 0.01	0.4 ± 0.02	0.4 ± 0.02	0.4 ± 0.03	0.4 ± 0.02
16:1n-7	7.2 ± 0.09	14.3 ± 0.71	14.6 ± 0.74	10.9 ± 0.65	11.3 ± 0.67
16:2n-4	0.3 ± 0.01	0.3 ± 0.02	0.3 ± 0.01	0.4 ± 0.03	0.3 ± 0.02
16:3n-6	0.8 ± 0.01	0.4 ± 0.03	0.6 ± 0.03	0.4 ± 0.04	0.7 ± 0.04
16:3n-4	0.7 ± 0.01	0.2 ± 0.03	0.3 ± 0.03	0.2 ± 0.04	0.4 ± 0.04
16:4n-1	0.9 ± 0.02	0.3 ± 0.05	0.4 ± 0.04	0.3 ± 0.06	0.5 ± 0.05
18:0	3.7 ± 0.07	1.4 ± 0.18	1.5 ± 0.13	1.9 ± 0.38	2.2 ± 0.21
18:1n-11	0.3 ± 0.01	3.7 ± 0.63	2.5 ± 0.23	3.3 ± 0.75	2.1 ± 0.29
18:1n-9	10.9 ± 0.05	16.8 ± 0.95	17.7 ± 0.74	16.6 ± 1.24	17.4 ± 0.83
18:1n-7	3.0 ± 0.02	4.8 ± 0.26	4.7 ± 0.23	4.7 ± 0.36	4.4 ± 0.24
18:1n-5	0.2 ± 0.01	0.5 ± 0.01	0.4 ± 0.02	0.4 ± 0.01	0.3 ± 0.02
18:2n-6	1.3 ± 0.01	1.4 ± 0.04	1.6 ± 0.05	1.5 ± 0.07	1.7 ± 0.06
18:3n-3	1.0 ± 0.01	0.6 ± 0.03	0.8 ± 0.04	0.6 ± 0.06	0.9 ± 0.06
18:4n-3	2.2 ± 0.03	1.0 ± 0.09	1.2 ± 0.08	0.9 ± 0.13	1.3 ± 0.10
20:0	0.3 ± 0.01	0.0 ± 0.01	0.0 ± 0.01	0.1 ± 0.01	0.1 ± 0.01
20:1n-11	0.5 ± 0.01	1.6 ± 0.15	1.3 ± 0.06	1.7 ± 0.20	1.2 ± 0.06
20:1n-9	4.0 ± 0.18	6.3 ± 0.85	5.3 ± 0.45	7.1 ± 1.26	5.4 ± 0.58
20:1n-7	0.4 ± 0.01	0.6 ± 0.06	0.5 ± 0.06	0.8 ± 0.12	0.6 ± 0.10
20:2n-6	0.3 ± 0.00	0.3 ± 0.02	0.2 ± 0.01	0.3 ± 0.02	0.2 ± 0.02
20:4n-6	1.0 ± 0.01	0.7 ± 0.12	0.7 ± 0.05	0.8 ± 0.23	0.8 ± 0.07
20:4n-3	0.7 ± 0.01	0.5 ± 0.03	0.6 ± 0.03	0.5 ± 0.04	0.7 ± 0.04
20:5n-3	11.2 ± 0.11	5.2 ± 0.34	5.7 ± 0.25	4.6 ± 0.36	5.4 ± 0.38
22:1n-11	4.5 ± 0.17	1.9 ± 0.66	1.4 ± 0.38	2.8 ± 1.00	1.9 ± 0.47
22:1n-9	0.5 ± 0.02	0.3 ± 0.07	0.3 ± 0.04	0.4 ± 0.12	0.3 ± 0.06
21:5n-3	0.6 ± 0.01	0.4 ± 0.01	0.4 ± 0.01	0.4 ± 0.02	0.5 ± 0.01
22:5n-6	0.3 ± 0.00	0.3 ± 0.01	0.3 ± 0.01	0.3 ± 0.02	0.3 ± 0.02
22:5n-3	2.1 ± 0.02	5.5 ± 0.30	5.1 ± 0.31	6.1 ± 0.50	5.3 ± 0.48
22:6n-3	11.1 ± 0.09	12.0 ± 0.63	11.2 ± 0.50	12.5 ± 0.86	10.9 ± 0.64

Spring 2000A

Fatty Acid	Diet Average n = 16	Blubber Samples Initial n = 10	Final n = 10	Initial Inner n = 10	Final Inner n = 10
14:0	7.0 ± 0.02	4.3 ± 0.08	4.9 ± 0.13	4.6 ± 0.13	5.7 ± 0.22
14:1n-5	0.1 ± 0.00	1.1 ± 0.05	1.1 ± 0.04	0.8 ± 0.06	0.8 ± 0.04
15:0	0.4 ± 0.00	0.3 ± 0.01	0.3 ± 0.01	0.3 ± 0.01	0.3 ± 0.01
16:0	13.0 ± 0.03	8.3 ± 0.17	8.5 ± 0.18	8.7 ± 0.26	9.1 ± 0.26
16:1n-11	0.3 ± 0.00	0.5 ± 0.02	0.5 ± 0.01	0.4 ± 0.02	0.4 ± 0.01
16:1n-9	0.2 ± 0.00	0.4 ± 0.02	0.4 ± 0.01	0.4 ± 0.03	0.4 ± 0.02
16:1n-7	6.7 ± 0.02	14.7 ± 0.29	14.1 ± 0.33	11.8 ± 0.28	10.9 ± 0.32
16:2n-6	0.1 ± 0.00	0.1 ± 0.00	0.1 ± 0.00	0.1 ± 0.00	0.1 ± 0.00
16:2n-4	0.2 ± 0.00	0.3 ± 0.02	0.2 ± 0.01	0.3 ± 0.03	0.2 ± 0.02
16:3n-6	0.6 ± 0.00	0.5 ± 0.02	0.6 ± 0.01	0.5 ± 0.02	0.6 ± 0.02
16:3n-4	0.5 ± 0.00	0.3 ± 0.02	0.3 ± 0.02	0.3 ± 0.01	0.4 ± 0.01
16:4n-1	1.2 ± 0.01	0.5 ± 0.03	0.6 ± 0.03	0.6 ± 0.05	0.7 ± 0.04
18:0	1.4 ± 0.01	1.0 ± 0.05	1.0 ± 0.04	1.4 ± 0.09	1.3 ± 0.06
18:1n-11	0.3 ± 0.00	4.5 ± 0.35	4.4 ± 0.42	3.8 ± 0.44	3.9 ± 0.37
18:1n-9	9.6 ± 0.01	16.1 ± 0.66	17.5 ± 0.63	14.7 ± 0.73	17.4 ± 0.50
18:1n-7	2.5 ± 0.01	4.8 ± 0.19	4.6 ± 0.15	4.5 ± 0.20	4.2 ± 0.13
18:1n-5	0.4 ± 0.00	0.5 ± 0.01	0.5 ± 0.01	0.5 ± 0.01	0.4 ± 0.01
18:2n-6	1.1 ± 0.00	1.4 ± 0.06	1.5 ± 0.06	1.3 ± 0.05	1.6 ± 0.05
18:3n-3	0.6 ± 0.00	0.4 ± 0.01	0.6 ± 0.02	0.4 ± 0.02	0.6 ± 0.04
18:4n-3	2.8 ± 0.01	0.9 ± 0.03	1.2 ± 0.06	0.9 ± 0.03	1.4 ± 0.11
20:0	0.1 ± 0.00	0.1 ± 0.00	0.0 ± 0.00	0.1 ± 0.01	0.1 ± 0.00
20:1n-11	0.6 ± 0.01	2.3 ± 0.17	2.4 ± 0.18	2.4 ± 0.19	2.6 ± 0.19
20:1n-9	11.8 ± 0.05	7.2 ± 0.51	8.3 ± 0.43	7.8 ± 0.56	9.7 ± 0.46
20:1n-7	0.5 ± 0.00	0.7 ± 0.05	0.6 ± 0.04	0.8 ± 0.07	0.6 ± 0.04
20:2n-6	0.2 ± 0.00	0.2 ± 0.01	0.2 ± 0.01	0.2 ± 0.01	0.2 ± 0.01
20:4n-6	0.4 ± 0.00	0.5 ± 0.04	0.4 ± 0.03	0.5 ± 0.04	0.4 ± 0.02
20:4n-3	0.4 ± 0.00	0.5 ± 0.02	0.5 ± 0.02	0.5 ± 0.01	0.6 ± 0.03
20:5n-3	9.5 ± 0.03	5.1 ± 0.22	4.8 ± 0.19	5.2 ± 0.31	4.5 ± 0.25
22:1n-11	15.1 ± 0.09	2.0 ± 0.27	2.2 ± 0.14	3.3 ± 0.51	3.4 ± 0.26
22:1n-9	1.4 ± 0.01	0.4 ± 0.04	0.4 ± 0.03	0.5 ± 0.06	0.5 ± 0.04
21:5n-3	0.4 ± 0.00	0.4 ± 0.01	0.4 ± 0.01	0.5 ± 0.02	0.4 ± 0.01
22:5n-6	0.2 ± 0.01	0.2 ± 0.00	0.2 ± 0.01	0.2 ± 0.01	0.2 ± 0.01
22:5n-3	0.7 ± 0.00	5.6 ± 0.11	4.9 ± 0.14	6.5 ± 0.18	4.8 ± 0.28
22:6n-3	6.1 ± 0.04	10.4 ± 0.41	8.7 ± 0.43	11.7 ± 0.53	8.1 ± 0.64

Spring 2000B

Fatty Acid	Diet Average n = 14	Blubber Samples			
		Initial n = 10	Final n = 10	Initial Inner n = 10	Final Inner n = 10
14:0	7.7 ± 0.02	5.0 ± 0.38	5.1 ± 0.29	6.1 ± 1.19	5.8 ± 0.43
14:1n-5	0.1 ± 0.00	1.0 ± 0.04	0.9 ± 0.05	0.7 ± 0.07	0.6 ± 0.05
15:0	0.4 ± 0.00	0.3 ± 0.01	0.3 ± 0.01	0.3 ± 0.02	0.3 ± 0.02
16:0	17.5 ± 0.07	9.4 ± 0.49	10.0 ± 0.55	10.6 ± 1.03	11.3 ± 0.80
16:1n-11	0.3 ± 0.00	0.5 ± 0.02	0.5 ± 0.02	0.4 ± 0.03	0.4 ± 0.02
16:1n-9	0.2 ± 0.00	0.4 ± 0.03	0.4 ± 0.02	0.4 ± 0.05	0.4 ± 0.03
16:1n-7	8.2 ± 0.02	13.6 ± 0.55	13.6 ± 0.51	11.3 ± 0.96	10.8 ± 0.31
16:2n-6	0.3 ± 0.00	0.1 ± 0.01	0.1 ± 0.01	0.1 ± 0.01	0.1 ± 0.01
16:2n-4	0.2 ± 0.00	0.3 ± 0.01	0.2 ± 0.01	0.3 ± 0.02	0.3 ± 0.01
16:3n-6	1.2 ± 0.01	0.5 ± 0.04	0.7 ± 0.05	0.5 ± 0.08	0.8 ± 0.08
16:3n-4	2.1 ± 0.01	0.3 ± 0.02	0.6 ± 0.06	0.3 ± 0.05	0.7 ± 0.10
16:4n-1	3.5 ± 0.02	0.6 ± 0.09	1.0 ± 0.12	0.7 ± 0.15	1.3 ± 0.19
18:0	3.0 ± 0.02	1.2 ± 0.07	1.3 ± 0.08	1.6 ± 0.11	1.8 ± 0.10
18:1n-11	0.1 ± 0.01	4.1 ± 0.35	3.0 ± 0.41	3.0 ± 0.43	2.1 ± 0.39
18:1n-9	11.2 ± 0.03	16.2 ± 1.22	17.6 ± 0.83	15.4 ± 1.87	17.4 ± 1.18
18:1n-7	2.8 ± 0.01	4.3 ± 0.29	4.4 ± 0.22	3.8 ± 0.41	4.1 ± 0.24
18:1n-5	0.1 ± 0.00	0.5 ± 0.01	0.4 ± 0.02	0.4 ± 0.03	0.3 ± 0.03
18:2n-6	0.8 ± 0.00	1.2 ± 0.05	1.3 ± 0.05	1.2 ± 0.09	1.3 ± 0.06
18:3n-3	0.5 ± 0.00	0.5 ± 0.04	0.5 ± 0.04	0.5 ± 0.06	0.6 ± 0.04
18:4n-3	2.5 ± 0.01	1.0 ± 0.10	1.2 ± 0.09	1.0 ± 0.17	1.2 ± 0.12
20:0	0.2 ± 0.00	0.1 ± 0.00	0.1 ± 0.00	0.1 ± 0.01	0.1 ± 0.00
20:1n-11	0.1 ± 0.00	2.0 ± 0.14	1.6 ± 0.15	1.9 ± 0.22	1.5 ± 0.25
20:1n-9	1.0 ± 0.01	6.9 ± 0.34	5.9 ± 0.44	6.9 ± 0.71	5.5 ± 0.76
20:1n-7	0.2 ± 0.00	0.5 ± 0.06	0.5 ± 0.05	0.5 ± 0.10	0.5 ± 0.08
20:2n-6	0.2 ± 0.00	0.2 ± 0.01	0.2 ± 0.01	0.2 ± 0.02	0.2 ± 0.01
20:4n-6	1.2 ± 0.00	0.6 ± 0.04	0.6 ± 0.03	0.6 ± 0.08	0.7 ± 0.05
20:4n-3	0.6 ± 0.00	0.5 ± 0.03	0.6 ± 0.04	0.5 ± 0.05	0.6 ± 0.04
20:5n-3	18.4 ± 0.07	5.5 ± 0.57	6.5 ± 0.52	5.5 ± 0.88	6.9 ± 0.77
22:1n-11	0.7 ± 0.02	2.1 ± 0.44	1.3 ± 0.25	3.1 ± 0.75	1.7 ± 0.41
22:1n-9	0.1 ± 0.00	0.3 ± 0.03	0.3 ± 0.03	0.4 ± 0.07	0.3 ± 0.05
21:5n-3	0.8 ± 0.00	0.4 ± 0.02	0.5 ± 0.02	0.4 ± 0.04	0.5 ± 0.03
22:5n-3	1.8 ± 0.01	5.5 ± 0.20	5.4 ± 0.12	5.7 ± 0.37	5.7 ± 0.20
22:6n-3	7.8 ± 0.03	10.8 ± 0.39	9.7 ± 0.40	11.9 ± 0.77	9.8 ± 0.57

Appendix 2

Model estimates (mean ± SE) of the contribution of prey species to diets

Fall 1999:

	Model Estimates			
	Initial Whole n = 8	Final Whole n = 8	Initial Inner n = 8	Final Inner n = 8
Flounders				
American Plaice	0.0 ± 0.01	0.0 ± 0.09	0.0 ± 0.00	0.0 ± 0.00
Windowpane Flounder	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00
Winter Flounder	6.6 ± 3.28	5.5 ± 3.12	6.1 ± 3.20	3.3 ± 2.19
Witch Flounder	4.1 ± 2.79	4.2 ± 3.15	4.4 ± 3.18	3.6 ± 2.76
Yellowtail Flounder	0.5 ± 1.42	1.2 ± 1.40	0.0 ± 0.79	0.0 ± 0.00
Forage Fish				
Capelin	0.5 ± 0.92	0.0 ± 0.00	1.3 ± 2.00	0.0 ± 0.04
Gaspereau	0.0 ± 0.30	0.0 ± 0.18	0.3 ± 0.35	0.4 ± 0.40
Herring	3.8 ± 2.95	0.0 ± 0.31	12.2 ± 7.96	0.2 ± 0.86
Mackerel	0.0 ± 0.16	0.0 ± 0.00	0.4 ± 0.50	0.0 ± 0.03
Northern Sandlance	17.4 ± 7.12	27.2 ± 8.87	6.5 ± 5.19	24.6 ± 7.81
Snake Blenny	0.0 ± 0.30	0.0 ± 0.22	0.0 ± 0.00	0.0 ± 0.17
Gadids				
Cod	4.3 ± 3.35	5.1 ± 4.03	1.0 ± 1.32	0.0 ± 0.00
Haddock	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00
Pollock	0.0 ± 0.01	0.0 ± 0.00	1.4 ± 1.47	2.1 ± 2.14
Red Hake	6.1 ± 4.46	0.1 ± 0.92	2.5 ± 2.70	0.0 ± 0.00
Silver Hake	0.0 ± 0.28	0.0 ± 0.01	0.0 ± 0.07	0.0 ± 0.00
White Hake	0.0 ± 0.68	0.0 ± 0.71	3.0 ± 3.85	0.0 ± 0.00
Skates				
Thorny Skate	0.0 ± 0.00	0.0 ± 0.02	0.0 ± 0.00	0.0 ± 0.00
Winter Skate	15.8 ± 4.62	14.7 ± 5.24	18.5 ± 6.69	24.1 ± 8.52
Other Fish				
Longhorn Sculpin	6.1 ± 4.46	0.1 ± 0.92	2.5 ± 2.70	0.0 ± 0.00
Ocean Pout	0.0 ± 0.13	0.0 ± 0.00	0.3 ± 0.70	0.0 ± 0.11
Redfish	26.7 ± 10.62	20.2 ± 7.19	34.3 ± 12.31	26.6 ± 8.61
Invertebrates				
Lobster	0.0 ± 0.01	0.0 ± 0.00	1.4 ± 1.47	2.1 ± 2.14
Northern Shrimp	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00
Rock Crab	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00
Experimental Diet	1.5 ± 0.68	11.6 ± 2.90	0.1 ± 0.12	13.8 ± 4.53

Spring 2000A

	Model Estimates			
	Initial Whole n = 10	Final Whole n = 10	Initial Inner n = 10	Final Inner n = 10
Flounders				
American Plaice	3.4 ± 3.85	0.0 ± 1.13	0.0 ± 0.48	0.0 ± 0.02
Windowpane Flounder	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00
Winter Flounder	0.6 ± 0.70	0.2 ± 0.41	0.4 ± 0.63	0.0 ± 0.11
Witch Flounder	2.1 ± 1.85	2.1 ± 1.62	4.1 ± 2.79	1.9 ± 1.59
Yellowtail Flounder	8.6 ± 4.88	4.6 ± 2.54	4.4 ± 3.15	0.0 ± 0.00
Forage Fish				
Capelin	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.04
Gaspereau	0.0 ± 0.04	0.1 ± 0.33	0.0 ± 0.16	0.8 ± 0.86
Herring	3.1 ± 2.12	0.0 ± 0.37	14.8 ± 4.17	9.7 ± 4.77
Mackerel	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00
Northern Sandlance	24.7 ± 7.68	18.7 ± 5.63	18.1 ± 5.33	4.4 ± 2.29
Snake Blenny	0.6 ± 0.68	1.1 ± 1.13	0.0 ± 0.16	0.0 ± 0.00
Gadids				
Cod	3.1 ± 3.33	0.0 ± 0.15	0.0 ± 0.17	0.0 ± 0.00
Haddock	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00
Pollock	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00
Red Hake	0.1 ± 0.87	0.0 ± 0.20	0.0 ± 0.03	0.0 ± 0.00
Silver Hake	2.9 ± 3.17	0.0 ± 0.72	0.0 ± 0.03	0.0 ± 0.00
White Hake	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00
Skates				
Thorny Skate	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00
Winter Skate	6.5 ± 2.93	14.4 ± 3.83	5.9 ± 2.93	7.5 ± 3.43
Other Fish				
Longhorn Sculpin	0.1 ± 0.87	0.0 ± 0.20	0.0 ± 0.03	0.0 ± 0.00
Ocean Pout	0.0 ± 0.04	0.0 ± 0.03	0.0 ± 0.01	0.0 ± 0.00
Redfish	33.3 ± 7.11	41.0 ± 8.39	45.3 ± 7.73	61.6 ± 6.83
Invertebrates				
Lobster	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00
Northern Shrimp	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00
Rock Crab	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00
Experimental Diet	1.9 ± 0.76	8.1 ± 2.65	0.5 ± 0.42	13.4 ± 5.83

Spring 2000B

	Model Estimates			
	Initial Whole n = 10	Final Whole n = 10	Initial Inner n = 10	Final Inner n = 10
Flounders				
American Plaice	0.0 ± 0.73	0.0 ± 1.43	0.0 ± 0.10	0.0 ± 0.45
Windowpane Flounder	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00
Winter Flounder	0.2 ± 0.38	0.2 ± 0.43	0.2 ± 0.31	0.4 ± 1.01
Witch Flounder	2.8 ± 1.70	9.0 ± 2.88	1.3 ± 1.10	7.1 ± 2.81
Yellowtail Flounder	0.0 ± 0.38	0.0 ± 1.16	0.0 ± 0.08	0.0 ± 0.47
Forage Fish				
Capelin	0.0 ± 0.00	0.0 ± 0.00	0.9 ± 1.06	0.0 ± 0.00
Gaspereau	1.0 ± 0.97	0.0 ± 0.08	3.4 ± 2.36	0.3 ± 0.37
Herring	2.1 ± 2.14	0.3 ± 0.75	5.1 ± 3.43	0.9 ± 1.68
Mackerel	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00
Northern Sandlance	21.7 ± 7.97	25.5 ± 8.13	19.1 ± 8.01	21.7 ± 7.64
Snake Blenny	0.1 ± 0.20	0.0 ± 0.07	0.0 ± 0.13	0.0 ± 0.00
Gadids				
Cod	1.8 ± 1.84	0.0 ± 0.18	0.0 ± 0.02	0.0 ± 0.00
Haddock	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00
Pollock	0.0 ± 0.03	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00
Red Hake	0.2 ± 0.94	4.1 ± 3.55	0.0 ± 0.00	0.0 ± 0.09
Silver Hake	0.0 ± 1.55	3.7 ± 3.29	0.0 ± 0.11	0.0 ± 0.74
White Hake	1.5 ± 2.03	0.9 ± 1.65	5.9 ± 5.27	1.1 ± 1.71
Skates				
Thorny Skate	0.0 ± 0.03	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00
Winter Skate	16.9 ± 5.36	14.3 ± 4.88	21.7 ± 7.42	21.4 ± 8.45
Other Fish				
Longhorn Sculpin	0.2 ± 0.94	4.1 ± 3.55	0.0 ± 0.00	0.0 ± 0.09
Ocean Pout	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.08
Redfish	32.9 ± 4.47	26.7 ± 3.12	37.0 ± 8.11	31.1 ± 5.92
Invertebrates				
Lobster	0.0 ± 0.03	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00
Northern Shrimp	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00
Rock Crab	0.0 ± 0.00	0.0 ± 0.25	0.0 ± 0.00	0.0 ± 0.12
Experimental Diet	2.3 ± 0.29	9.0 ± 1.91	0.7 ± 0.28	13.8 ± 5.00

Chapter 6. General Conclusions

Throughout this research, I investigated a number of aspects of FA metabolism in juvenile pinnipeds and developed techniques that will be useful in future studies. I have shown that, despite their large body size, it is possible to study the modification and deposition of specific dietary FA in pinnipeds directly. A particularly interesting application of this technique will be to study the fate of dietary n-3 FA in these animals. Since pinnipeds typically consume large amounts of the long-chain n-3 PUFA, unlike most animals previously studied, unique patterns of deposition and interconversion may be found. My *in vivo* labelling studies have also shown that mink are a good animal model to investigate the metabolism of marine lipids by a carnivore accustomed to their consumption. Future research in which mink will be useful is the refinement of calibration coefficients applicable to predators that store TAG in adipose tissue as opposed to blubber, such as polar bears. Obviously controlled feeding studies involving polar bears would be extremely difficult and costly and mink will provide an excellent alternative.

My research demonstrated that although individual dietary FA experience differential metabolism leading up to their incorporation into chylomicrons the chylomicron FA signatures of grey seals resemble that of a recent meal. More important, however, is the finding that chylomicron FA signatures can be used to make accurate, quantitative predictions of the prey composition of a recent meal in a marine carnivore. Although further work should examine mixed-species diets, this is the first time that diet has been studied, in a quantitative fashion, using the FA composition of chylomicrons. It

is clear from this work that to use chylomicron FA signatures to estimate diet, chylomicrons must be visibly present and isolated from the rest of the blood sample prior to FA analysis. If whole blood, plasma or serum were analyzed the increased input of endogenous FA, carried in the other lipoprotein classes, would lead to highly erroneous results in the modeling of diets. Better yet, would be to isolate TAG FA within the chylomicrons, which would likely provide an even more accurate estimate of diet.

I designed a means by which free-ranging seals can be brought into captivity for short-duration feeding experiments in which completely homogenous diets can be fed to individuals. The primary purpose of these studies was to further develop the QFASA method using precise knowledge of the FA composition consumed by the seals. However, because this method of feeding had not been evaluated, an important goal was to determine how animals would respond. Considering the adverse health effects exhibited by many of the seals, and the apparently low utilization of the energy in the homogenous diets, this feeding method proved less than ideal. It is simply not possible to intubate sufficient amounts of feed (number of meals and size of each meal) in these animals on a daily basis to achieve adequate mass and fat gain without undo stress. Despite this, the results from these controlled feeding studies demonstrate that even over a relatively brief period of time, FA signatures of a new diet are reflected in predator blubber stores and that as long the differential metabolism of FA within the predator is accounted for, blubber FA can be used to provide an accurate quantitative estimation of diet composition using QFASA.

A limitation of my experimental design was that, because my study animals were wild seals, the diet consumed prior to the experimental period could not be known. This

created two problems. First, it complicated the interpretation of changes in blubber FA composition that occurred over the course of the experiment. Although a greater similarity between the level of a FA in the final blubber signatures and the experimental diet signature implied a shift in the direction of the diet, without being able to compare the levels of individual FA between the control and experimental diets as well as between the initial and final blubber FA signatures, this cannot be said with certainty. Second, it prevented the use of a control group in the experiments. To truly show that the changes observed in the FA composition of the blubber of my study animals were a result of a change in diet, I would have had to feed control animals the same diet that they were consuming prior to the experiment. This was not possible given the constraints of this field situation and the animals studied.

Through these controlled feeding studies I was also able to begin to address the question of the time frame over which FA signatures are integrating diet. It appears that while whole blubber provides a longer-term integration of the dietary history of an individual, modeling the inner half of the blubber layer provides a view of more recent diet. The *in vivo* radio-labelled FA tracer method described here and employed with a wider array of FA would likely prove very useful in future studies of blubber FA turnover rates and the assessment of time frames over which diets can be inferred.

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