Review Article

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STUDYING TROPHIC ECOLOGY IN MARINE ECOSYSTEMS USING FATTY ACIDS: A PRIMER ON ANALYSIS AND INTERPRETATION

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Fatty acids (FA) represent a large group of molecules that comprise the majority of lipids found in all organisms. Their great diversity, biochemical restrictions and, in some cases, unique origin among plants and animals has fostered a number of areas of research, ranging from assessment of animal nutrition and metabolism, to investigating trophic interactions and ecosystem structure. Over the past three decades, we have observed the use of FA develop from a potential tool for delineating food webs (Ackman and Eaton 1966) to a powerful technique for quantitative assessment of predator diets (Iverson *et al*. 2004). Studies that have compared the FA found in predator fat stores with those found in their prey have allowed both qualitative (*e.g.*, Horgan and Barrett 1985; Smith *et al*. 1996; Raclot *et al*. 1998; Dahl *et al*. 2000, 2003; Falk-Petersen *et al*. 2004) and quantitative or semi-quantitative analyses of diet (Kirsch *et al*. 2000; Iverson *et al*. 2001*b*, 2004; Iverson and Springer 2002) and

comparisons of the spatial and temporal scales of foraging (*e.g.*, Iverson *et al.* 1997*a*, *b*; Smith *et al.* 1997; Walton *et al.* 2000). These studies are possible because the FA consumed by a monogastric (*i.e.*, single-chambered stomach) predator are deposited into adipose tissue with little modification or in a predictable pattern, thus providing an integrated record of dietary intake over time. Previous studies, together with the recent development of quantitative FA signature analysis (QFASA; Iverson *et al.* 2004), have generated considerable interest in the use of FA to study the foraging ecology and diets of upper trophic level predators, such as marine mammals, bears and seabirds. Given that the use of FA as an ecological tool is a relatively new and rapidly developing field, it is inevitable that a variety of methods have been used both to chemically analyze FA and to interpret the resulting data. In both of these areas, we believe there is some confusion in the literature. Furthermore, many investigators from around the world frequently inquire about the requirements and limitations of using FA to address ecological questions. Given this broad interest, particularly among researchers studying marine mammals, we felt it was both necessary and timely to review appropriate methods for the chemical analysis and interpretation of FA in the context of ecological research. Although we focus on applications involving marine mammals and seabirds, most of the issues we discuss are pertinent to other marine and terrestrial predators and the methods should be broadly applicable to other taxa.

FA can be used to study foraging ecology and food webs in three ways. First, by examining changes in FA distributions, or "signatures" (Iverson 1993) of the predator alone, we can ask qualitative questions about spatial or temporal variations in diets, both among and within individuals or populations (*e.g.*, Iverson *et al.* 1997*a*, *b*; Walton *et al.* 2000; Beck *et al.* 2005). The second use of FA is also qualitative, but relies on the use of individual biomarkers or tracers—that is, the existence of a unique FA found in a predator that can be traced to a single origin or prey species (*e.g.*, Pascal and Ackman 1975, reviewed in Iverson 1993). In principle, this opportunity will be relatively rare, as FA present in either marine or terrestrial environments are relatively ubiquitous within those environments. However, this biomarker approach can be extended; for instance, unusual levels of certain FA or of ratios among FA that can only be attributed to one or a few prey types may indicate their likely unimportance or dominance in the diet (reviewed in Dalsgaard *et al.* 2003). The final and most ambitious way in which to use FA is to quantitatively estimate diet from FA signatures of predator and prey. The QFASA method (Iverson *et al.* 2004) uses a statistical model to compute the most likely combination of prey FA signatures that comes closest to matching that observed in the predator, after accounting for predator FA metabolism by mathematically weighting individual FA. This procedure requires knowledge of the FA compositions of all important potential prey species, as well as sufficient within-species sampling to assess variability or overlap in signatures with ecological and demographic factors (*e.g.*, Budge *et al.* 2002, Iverson *et al.* 2002). Each of these uses of FA can provide somewhat different, but valuable information on the foraging ecology of free-ranging predators that are difficult to observe or access over much of their spatial and temporal ranges, and for which existing methods, such as fecal or stomach contents analysis, may provide biased information. Nevertheless, all of the methods using FA require knowledge of the underlying biochemistry and the appropriate techniques to ensure both accurate chemical analysis of FA composition and appropriate interpretation of the resulting data.

The aim of this review is to provide investigators with a guide that describes the suite of requirements and tools necessary to conduct ecological studies using FA. We concentrate on issues related to the use of FA for studying trophic interactions or

diet in upper trophic level marine predators, although we also touch upon other ways in which to use FA in ecological studies. We begin by providing a description of basic lipid structures and their biochemistry as these relate to diet and biosynthesis. We then discuss appropriate sampling, storage and chemical analysis, and highlight common and potential problems. We also describe the basic gas chromatographic (GC) instrumentation and software necessary for analysis, and briefly discuss some statistical methods currently used to analyze and interpret the resulting data. Lastly, we discuss taxon-specific issues in the use of FA.

Overview of Lipid and FA Structure and Metabolism

Lipid Structures

Lipids are a heterogeneous group of compounds that share the common property of being insoluble in water but soluble in organic solvents such as chloroform, hydrocarbons and alcohols. The most abundant lipids are those that contain FA as part of their structure. FA most commonly consist of an even-numbered, straight carbon chain, containing 14 to 24 carbons and zero to six double bonds, with a methyl terminus (CH_3) at one end and an acid (carboxyl) terminus at the other (Fig. 1).

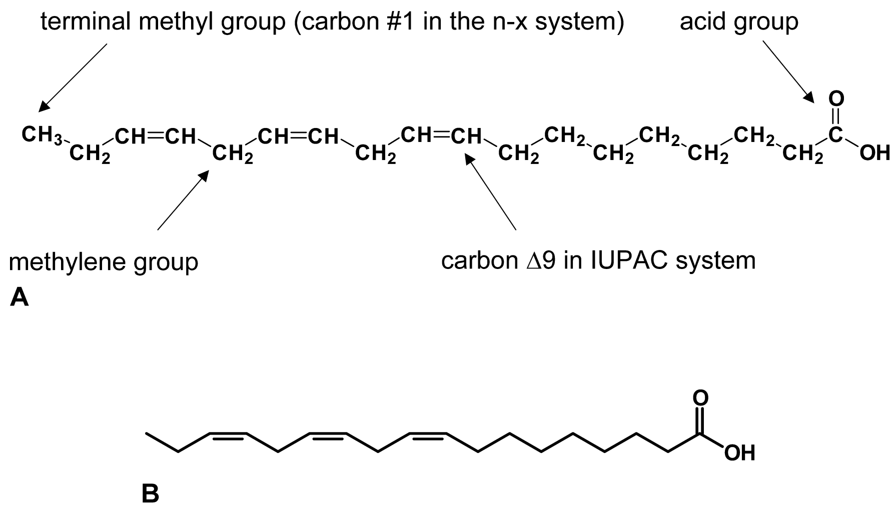


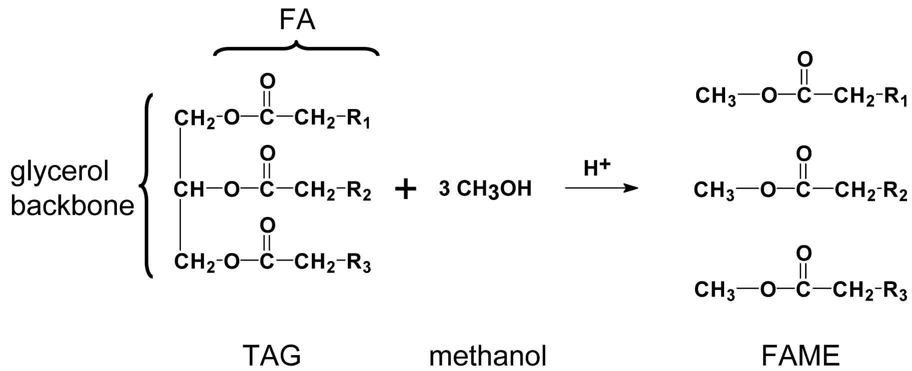
Figure 1. Example of FA structure and nomenclature. (A) The structure of 18:3n-3 with all atoms indicated; and (B) the same FA structure with carbon and hydrogen atoms assumed. FA are named using shorthand notation of A:Bn-X, where A indicates the number of carbon atoms, B is the number of double bonds and X indicates the position of the first double bond relative to the terminal methyl group (*i.e.*, 18:3n-3). With this system, it is assumed that each double bond is separated by a methylene group (CH_2 group). Some investigators use the slightly shorter notation of A:BωX (*i.e.*, 18:3ω3), which has the same meaning but is simply an older format. This notation has developed because of metabolic relationships; once introduced into a FA, organisms have very limited ability to alter the position of the double bond closest to the terminal methyl group. A less common notation, that follows the recommendations of the International Union of Pure and Applied Chemistry (IUPAC), gives the position of double bonds relative to the acid group (COOH) and uses the Δ symbol (*i.e.*, 18:3Δ9,12,15). This system has the advantage of using the same naming conventions as FA desaturase enzymes; however, the n-x system is more useful in tracking families of FA with similar structures.

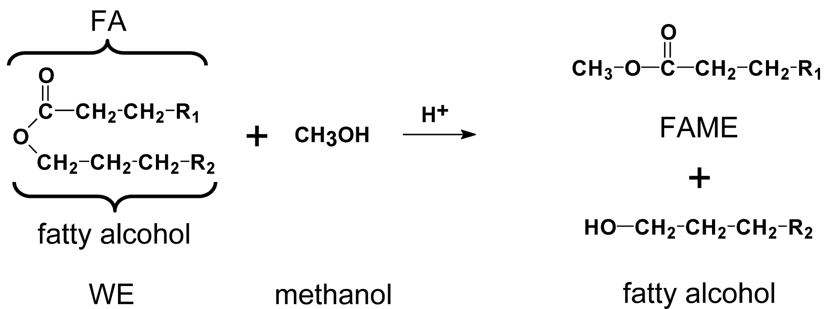
Nevertheless, FA in certain food sources can be as short as 4–6 carbons (*e.g.*, milks of ruminant mammals) and 8–10 carbons (*e.g.*, milks of rodents, lagomorphs and primates; some seed lipids) (Iverson and Oftedal 1992). However, in marine and terrestrial food webs involving upper trophic level carnivores, the shortest FA that is generally present above trace amounts is 12 carbons in length, but this still usually accounts for $\leq$0.1% of total FA. Extremely short branched-chain FA are found in structural and acoustic tissues of odontocetes, but these are not related to diet (see below). At the other extreme, FA with >24 carbon atoms and >6 double bonds also exist, but only in trace amounts as intermediates in most organisms (Voss *et al.* 1991). Thus, FA of 14 carbons with no double bonds to 24 carbons with 6 double bonds represent the range in length and unsaturation that is relevant in the study of most predators.

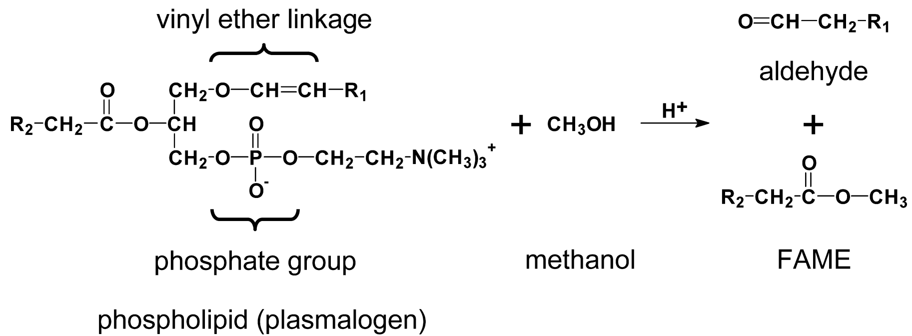
FA are most commonly named using a shorthand notation of A:Bn-*x*, where A indicates the number of carbon atoms, B is the number of double bonds and *x* indicates the position of the first double bond relative to the terminal methyl group (Fig. 1A). With this naming system, it is assumed that all FA are methylene-interrupted (*i.e.*, each double bond is separated by a -CH_2- group), which is by far their most common form. However, unusual FA do occur with double bonds that are non-methylene interrupted (NMI). Their presence in the food chain is due to synthesis by marine invertebrates, such as bivalve molluscs (reviewed in Joseph 1982). FA with zero and one double bond are known as saturated (SFA) and monounsaturated FA (MUFA), respectively. FA with two or more double bonds are known as polyunsaturated FA (PUFA). Lastly, some FA contain a methyl branch on the second or third carbon atom closest to the terminal methyl group. A methyl branch at the second carbon is indicated by prefacing the FA name with an "*i*" representing "iso"; "*ai*" (anti-iso) indicates a methyl branch at the third carbon (*e.g.*, *i*-15:0, *ai*-17:0).

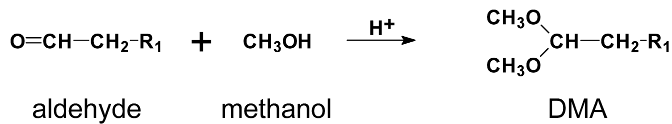
FA rarely exist in free form and are usually esterified to a glycerol backbone. Any lipid containing an esterified FA is called an acyl lipid. Triacylglycerols (TAG) represent the common form of storage lipids and make up the majority of lipids found in adipose tissue and blubber. TAG consist of three FA molecules esterified to a glycerol backbone (Fig. 2A). Animals mobilize stored TAG if FA requirements are not met through the diet or, conversely, deposit TAG when dietary FA and energy intake exceed demands. Thus, the composition of TAG FA in major lipid storage depots is relatively dynamic and most readily influenced by diet.

Figure 2. Lipid structures and FA derivatives produced using acidic transesterification. (A) A TAG consists of three FA moieties and a glycerol backbone. The reaction of TAG with MeOH in the presence of acid cleaves the FA from the backbone and forms the corresponding FAME; (B) WE, consisting of a FA esterified to a fatty alcohol, react with MeOH and acid in a similar manner to produce a FA and a fatty alcohol; and (C) A PL is similar to a TAG but contains a phosphate group attached to the third carbon of the glycerol backbone. The phosphate group may be bonded to a variety of derivatives; in this case, an amine group, choline, is attached. The structure illustrated is also an example of a specific type of phospholipid, the plasmalogen. Plasmalogens have an ether linkage to a long carbon chain containing a double bond adjacent to the ether functionality; this ether structure takes the place of one of the two FA and reacts with MeOH and acid to form an aldehyde that reacts further to produce DMA.


FA
glycerol backbone
CH_2-O-C(=O)-CH_2-R_1
CH-O-C(=O)-CH_2-R_2
CH_2-O-C(=O)-CH_2-R_3
+ 3 CH_3OH
H^+
CH_3-O-C(=O)-CH_2-R_1
CH_3-O-C(=O)-CH_2-R_2
CH_3-O-C(=O)-CH_2-R_3
TAG
methanol
FAME
A


FA
C(=O)-CH_2-CH_2-R_1
O
CH_2-CH_2-CH_2-R_2
fatty alcohol
+ CH_3OH
H^+
CH_3-O-C(=O)-CH_2-CH_2-R_1
FAME
+
HO-CH_2-CH_2-CH_2-R_2
WE
methanol
fatty alcohol
B


vinyl ether linkage
CH_2-O-CH=CH-R_1
R_2-CH_2-C(=O)-O-CH
CH_2-O-P(=O)(O$^-$)-O-CH_2-CH_2-$N(CH_3)_3^+$
phosphate group
phospholipid (plasmalogen)
+ CH_3OH
H^+
O=CH-CH_2-R_1
aldehyde
+
R_2-CH_2-C(=O)-O-CH_3
methanol
FAME


O=CH-CH_2-R_1
+ CH_3OH
H^+
CH_3O
CH-CH_2-R_1
CH_3O
aldehyde
methanol
DMA
C

Wax esters (WE) are a second type of lipid relevant to diet studies. WE consist of a FA esterified to a fatty alcohol (Fig. 2B). In theory, both the FA and the fatty alcohol can have any structure, but WE almost always contain saturated or monounsaturated fatty alcohols, unlike the FA portion which may have a variety of polyunsaturated structures. The functions of these WE are uncertain, but, like TAG, they are thought to be involved in energy storage in certain species of crustaceans, fish, and marine mammals. For example, copepods (*e.g.*, *Calanus* spp.) store almost all their energy as WE (Sargent 1976), and some fish such as myctophids, barracudina (*Notolepis rissoi*), capelin (*Mallotus villosus*), and herring (*Clupea harengus*) store significant amounts of WE in their adipose tissues (Ackman *et al*. 1972; Ratnayake and Ackman 1979; Phleger *et al*. 1997, 1999). WE are also found in the melons and mandibular regions of echolocating toothed whales (Varanasi and Malins 1971, Litchfield *et al*. 1978) and comprise the majority of storage lipids in the blubber of Pacific beaked whales (*Berardius bairdi*) and sperm whales (*Physeter macrocephalus*; Litchfield *et al*. 1976, Koopman 2001).

A third common acyl lipid class is the phospholipid (PL) group. PL normally consists of two FA esterified to a glycerol molecule that also contains a polar derivative of phosphatidic acid (Fig. 2C). PL are the structural components of all cell membranes. Because of the specialized functions of these lipids and membranes, organisms tend to conserve FA in PL, making this lipid class relatively robust to dietary changes in higher order predators and thus not informative as an indicator of diet. A special group of PL, the plasmalogens, contains a mono- or di-unsaturated carbon chain attached through a vinyl-ether linkage to the glycerol molecule in place of one of the FA (Fig. 2C). Vinyl-ether linkages are distinct from other ether linkages because of the presence of a double bond adjacent to the ether group. These lipids are thought to be involved in a variety of processes ranging from antioxidant functions to buoyancy control (Paltauf 1994) and are found throughout the marine food web. In particular, plasmalogens are common in bivalves (Joseph 1982) and in the gills of crustaceans (Chapelle and Benson 1986); smaller amounts are also found in many species of fish (Ackman 1998).

Other classes of acyl lipids exist, including glycolipids, sphingolipids and ceramides, but these are relatively rare and contribute little to the overall FA composition of organisms. Harwood and Russel (1984) and Gunstone *et al*. (1986) provide comprehensive reviews of all lipid classes.

Metabolism of Ingested Lipid

A basic understanding of lipid biochemistry and metabolism is critical to the interpretation of FA patterns in predator lipid stores. The FA composition of predator lipid stores is the result of three metabolic sources: (1) unmodified dietary FA that are directly deposited in adipose tissue, (2) dietary FA that are modified at some point between absorption into the blood and deposition, and (3) endogenously derived FA arising from *de novo* synthesis in the predator. Thus, it is important to understand the potential relative input from each process in any given tissue. In monogastric predators such as marine mammals and seabirds, by far the greatest contributor to adipose tissue FA composition is direct deposition of FA from diet, so that the relative influence of diet on a predator's fat stores may often be readily apparent. However, there will also be contributions from the other two processes. Thus, predator FA will never exactly match those of its prey. An understanding of when and where these

differences in FA arise allows the investigator to select those FA that are most relevant to the question being posed.

Lipid digestion and products—As noted earlier, we confine our discussion of lipid metabolism in higher order vertebrate predators to monogastric (non-ruminant) animals because, in ruminants, the extensive foregut fermentation generally results in the complete degradation of ingested FA. We also address only specific issues needed to understand how predator FA relate to diet, as there are several excellent reviews of general lipid and FA metabolism (*e.g.*, Harwood and Russell, 1984, Cook 1991, Gurr and Harwood 1991).

In the simplest and most common scenario, a prey item contains only acyl lipids, such as TAG and PL. During digestion by the predator, ingested lipids are hydrolyzed (*i.e.*, ester bonds are broken) to yield free FA (FFA), monoacylglycerols, and, in the case of PL, lysophospholipids (a glycerol backbone with a phosphatic acid derivative and only one FA). These products pass through the mucosal wall of the small intestine, reform into acyl lipids (*i.e.*, TAG) and are transported by chylomicrons (*i.e.*, the lipoproteins which carry FA derived from diet, consisting primarily of TAG) in the blood to tissues. However, short- and medium-chain FA < 14 C are an exception, as they are not incorporated into chylomicrons, but are transported to the liver where they are immediately oxidized (Brindley 1991, Papamandjaris *et al*. 1998). Thus, FA < 14 C that are present in predator lipids can only have arisen from *de novo* biosynthesis within that predator and, therefore, have no relation to diet. Once circulating chylomicron-TAG arrive at the tissue, they are again hydrolyzed. FA are then either metabolized for energy or re-esterified into TAG for storage. Some FA are also used in structural tissues or can be transformed into hormones and secondary messengers. Although all tissues contain FA in membrane structures or in small lipid depots, it is adipose tissue (or blubber in the case of marine mammals) that is the principle FA storage site and it is composed primarily of TAG. Adipose tissue TAG are also the principle form of energy storage in animals, as animals have only a very limited capacity to store carbohydrate. All FA consumed in excess of immediate requirements are deposited in this reservoir. As noted above, other than FA < 14 C, most FA are deposited in the TAG reservoir of adipose tissue in similar proportions to that consumed in the diet. However, there are notable exceptions (Iverson *et al*. 2004) that are relatively predictable given our current knowledge of FA metabolism, as discussed below.

In the case of TAG digestion discussed above, esterified FA are consumed and esterified FA are deposited. A slightly more complicated process occurs with the consumption of prey that contain long-chain, non-acid functional groups, such as the fatty alcohols in WE. When an ingested WE is metabolized, it is first hydrolyzed to produce its constituent FA and fatty alcohol. Enzymes in the gastrointestinal tract then act to oxidize the fatty alcohol to its corresponding FA (*i.e.*, an equal number of C atoms with the same double bond positions). This FA then joins the total ingested pool of FA available for incorporation into TAG, and is transported in chylomicrons and deposited. Thus, if a predator stores its FA as TAG in adipose tissue, it will do so even if it consumes a diet high in WE.

In contrast, in predators that have evolved to use WE as an energy storage form, the reverse process occurs whereby FA consumed in the diet are reduced to the corresponding fatty alcohol, which is then incorporated into WE for subsequent storage. It is not known where this reduction occurs, but given the absence of WE in circulating blood, it seems likely that it takes place at the site of WE formation in storage tissue. There is evidence from invertebrates and rats that transformation

of FA to fatty alcohols is not random. The enzymes responsible for catalyzing the reduction of FA to fatty alcohols is specific for MUFA, while PUFA are rarely present as alcohols (Bandi and Mangold 1973, Sargent 1976). Thus, it is important to note two issues. First, the consumption and storage of WE are decoupled in animals: whether a predator consumes WE or TAG has nothing to do with whether it stores FA as WE or TAG; WE storage seems to be a phylogenetic trait (Sargent *et al*. 1976). Second, FA stored in a predator that deposits WE will not be representative of all FA ingested because some portion of the fatty alcohol component of the storage WE has been derived from the reduction of ingested FA. Thus, both FA and fatty alcohol components must be analyzed to recreate the original composition of FA ingested. Storage of WE is clearly more complex to consider than that of TAG but, assuming accurate quantification of ingested and deposited WE alcohols and all other acyl lipids, we can treat the combined alcohol and FA composition of the diet as a single FA pool that the organism draws upon to fill its adipose tissue. That is, whether a TAG or a WE is formed by the predator, the same starting material is used: FA (from either ingested FA or oxidized fatty alcohol). It is the fatty hydrocarbon chain that is pertinent to diet estimation, and, because fatty alcohols and FA are essentially equivalent, the differing functionality of each is irrelevant.

The vinyl-ether linked carbon chains in plasmalogens are also modified during digestion to yield FA. In the intestine, enzymatic oxidation of the vinyl-ether linkage produces a fatty aldehyde (Fig. 2C) that is subsequently oxidized to the corresponding FA. The situation with plasmalogens is further complicated because, with typical methods of analysis, the vinyl-ether linkage is transformed into a dimethyl acetal (DMA, Fig. 2C) that interferes with the identification of some common FA. Because plasmalogens and other FA precursors, such as WE, ultimately add to the predator's FA pool, their levels in the prey must be determined. Studies that claim to "clean-up" their samples prior to analysis by removing all compounds that are not FA are often discarding portions containing these important FA precursors.

FA modification and biosynthesis—With an understanding of the relatively direct transport of FA from ingestion to deposition in adipose tissue, the next issue to consider in relating FA to diet is the extent to which FA may be modified during this process, or synthesized *de novo*, by the predator. Slight modification within the carbon chain of FA may occur between ingestion and deposition. Birds and mammals are capable of elongating the carbon chain of dietary FA by two carbon units and adding double bonds (desaturation) (Cook 1991). However, these modifications are typically restricted to SFA and MUFA, and are inhibited during both fasting and the consumption of high-fat diets (usually defined as >30% of total calories from fat), particularly those high in long-chain PUFA. While fat contents of prey vary seasonally, it is likely that most pinnipeds, cetaceans, and seabirds consume a diet in the range of 5% fat or more, containing high levels of long-chain PUFA, which would equate to >30% of calories. For instance, an average herring, at 8% fat and 17% protein, would contain about 44% of calories as fat. Thus, FA that have been elongated and desaturated are unlikely to make a significant contribution to the adipose FA in predators consuming such species. A more significant modification process may be the chain shortening of some long-chain MUFA. In both marine mammals and seabirds, reduced deposition, relative to diet, of most isomers of 22:1 and 20:1 are consistently observed (Iverson and Springer 2002, Iverson *et al*. 2004). Recent work on the gray seal (*Halichoerus grypus*) has shown that this is due, as in other vertebrates, to peroxisomal chain shortening of those isomers (Cooper *et al*. 2006). The main products of this chain shortening are 18:1 isomers (Norseth and Christophersen

1978, Osmundsen *et al.* 1979); consistent with this, enhanced deposition of these isomers is typically encountered in marine mammal and seabird fat depots. Captive feeding studies on seals have shown that dietary radio-labeled 18:1n-9 is directly deposited without modification and unlikely to arise from *de novo* synthesis (Budge *et al.* 2004). Thus, the enhanced levels of 18:1 relative to diet likely arise from chain shortening of 22:1 and 20:1. However, the predictable relationship between the isomers 22:1, 20:1, and 18:1 allows us to account for their levels in fat depots of marine predators (Iverson *et al.* 2004, Cooper *et al.* 2006).

De novo synthesis of FA (*i.e.*, biosynthesis of FA from carbohydrate or protein precursors) may also occur within predators, but, like elongation and desaturation, it is limited to SFA and MUFA and inhibited during fasting or by high-fat diets. When fasting, animals do not synthesize or modify fat, but rather mobilize it and completely oxidize it for energy. Conversely, when a predator is consuming a diet containing adequate or excess fat, including all the essential FA (*i.e.*, long-c hain PUFA that are prevalent in marine ecosystems), there is no need to synthesize fat. In birds and mammals, *de novo* synthesis typically only occurs when the animal is consuming a low-fat (usually defined as about 10% of total calories) and especially high-carbohydrate diet. In the gray seal, the primary product of *de novo* synthesis is 16:0 (Cooper 2004); this agrees with data from terrestrial vertebrates (Volpe and Vagelos 1973, Brindley 1978), so we can reasonably assume that the same is likely true in other marine predators. Thus, *de novo* synthesis in the predator is unlikely to be responsible for enhanced proportions of any FA other than 16:0. In fact, in seals, 16:0 is often present at amounts lower than that found in diet (Iverson *et al.* 2004), again indicating that *de novo* synthesis is unlikely to be an important source of FA in non-fasting animals consuming a high-fat diet.

Differential mobilization of some individual FA during fasting has been reported in seabirds and lactating seals (Groscolas 1990, Iverson *et al.* 1995). The pattern of differential mobilization was consistent among the predators examined in that 20:5n-3 was differentially mobilized while 20:1n-9 was selectively retained. Raclot (2003) has suggested that differential mobilization is determined solely by polarity of the FA. However, this explanation seems too simplistic, as differential mobilization of other polar FA, such as 22:6n-3 and 20:4n-6, is not observed. Additionally, data from long-term fasting studies in both northern elephant seal (*Mirounga angustirostris*) and gray seal pups have shown no net change in overall FA composition with time (D. Noren, unpublished data; S. J. Iverson and W. D. Bowen, unpublished data). While further studies on long-term fasting will be important, the slight effects of short-term fasting on FA composition are unlikely to have significant impacts on diet estimation.

Often branched-chain and odd-carbon numbered straight chain FA are present in trace amounts in the adipose tissue of vertebrate predators. Many of these, particularly those of 14–18 carbons in length, are originally derived from bacteria (Sargent *et al.* 1987, Ackman 1989) and normally accumulate in prey and predator adipose tissue at levels <2% of total FA (Iverson *et al.* 1995, Raclot *et al.* 1998, Budge *et al.* 2002, Iverson *et al.* 2002). Their levels in prey items will fluctuate depending on consumption of detritus and the activity of bacteria present in the gut. Little is known about the metabolism of these compounds once they are ingested, but presumably they are treated like normal FA and are deposited in the adipose tissue as TAG; however, these FA are not particularly indicative of dietary FA intake because they may be synthesized in both the predator (by gut flora) and prey, and are generally only present in trace quantities.

The adipose tissues of a few taxonomic marine predator groups contain substantial amounts of short branched-chain FA (*e.g.*, *i*-4:0, *i*-5:0, *i*-10:0 and *i*-12:0). For example, certain families of cetaceans (delphinids, phocoenids, and monodontids) contain considerable levels of the branched chain FA *i*-5:0 (isovaleric acid). In the blubber and head fats of some species (*e.g.*, northern right whale dolphin, *Lissodelphis borealis*) this compound is the dominant FA, accounting for up to 40% of total FA (Koopman *et al*. 2003). These short branched-chain FA are not derived from bacteria but instead are synthesized *de novo* from amino acid precursors (Morii and Kaneda 1982). In the blubber, they are hypothesized to play a role in tissue pliability in cold water environments (Koopman *et al*. 2003) and in head fats, to function in sound reception and transmission (Varanasi and Malins 1971, Koopman *et al*. 2006). This *de novo* synthesis most likely takes place locally, in the tissues in which these FA are found, as any significant quantities found in the circulation would likely be toxic (Koopman *et al*. 2003). This *de novo* synthesis means that, like the longer branched-chain FA, these short branched-chain FA are not indicative of diet. Indeed, as discussed above, if consumed in diet they would be completely oxidized after consumption. Nevertheless, the high concentration of these molecules in the tissues of some groups of animals requires different methods of analysis (see "Short chain FA" below).

Tissue Sampling, Storage, and Chemical Analysis

Tissues

A number of different tissues, including adipose tissue, blubber, milk, and blood, may contain information about diet, with the particular tissue type chosen depending on the focus of the research and the limitations of sampling a particular group of animals. These tissues provide insight into diet over different temporal scales. Adipose tissue is composed of fat cells (adipocytes) and may be metabolic (*i.e.*, site of energy storage) or structural (*e.g.*, cushions in eye sockets and in the pads of the feet of mammalian quadrupeds) in nature (Pond 1998). Structural adipose tissue usually has a specific composition of lipids and other constituents (of endogenous or exogenous origin) for its function and experiences relatively little turnover; thus, structural adipose tissue will bear little resemblance to dietary lipid intake. Therefore, only adipose tissue that serves as a fat energy depot, such as subcutaneous or mysenteric fat, is useful in diet determination. Blubber is a specialized type of subcutaneous adipose tissue found in marine mammals, comprised of adipocytes held within an organized matrix of proteinaceous fiber (Pond 1998). Blubber has a number of functions, including thermal insulation, hydrodynamic streamlining, acting as a biological spring to reduce the cost of locomotion and serving as an energy storage depot (Ryg *et al*. 1988, Pabst 1996, Iverson 2002). Thus, blubber can have a stratified structure depending on whether it is located in an area that serves primarily structural functions or in an area that serves primarily as the site of energy storage. As in the case of adipose tissue, only the non-structural lipid components of blubber contain the most useful and potentially quantitative information on diet (see below).

Both non-structural adipose tissue and blubber contain lipids that have accumulated over time. Thus, their FA can provide an integrated record of dietary intake over a period of weeks to months, and perhaps longer in some species. For instance, in phocid seals that are capital breeders, animals dramatically deplete their blubber

fat reserves during lactation and again during the moult. Thus, the large lipid stores present early in the breeding season will be most influenced by feeding in the prior 4–6 mo spent preparing for reproduction (*e.g.*, Beck *et al.* 2003), with little contribution from feeding earlier in the year. Nevertheless, in non-breeding seals the appearance of new dietary FA is evident within 1–2 wk of a switch in diet (*e.g.*, Kirsch *et al.* 2000, Cooper 2004, Iverson *et al.* 2004) and radio-labeled FA are detected in both inner and outer layers of blubber within 12 h of feeding (Budge *et al.* 2004, Cooper 2004). In contrast, the adipose tissue of income breeding seabirds that have high turnover and metabolic rates will always likely reflect a combination of more recent, as well as longer-term feeding on the scale of weeks (Iverson and Springer 2002). However, the time frame of integration has only truly been investigated in a few species to date and, thus, much remains to be learned in this area.

The FA composition of milk also reflects diet, but the temporal nature of this information depends on the reproductive strategy of a particular species. Capital breeders, such as phocid seals and many baleen whales, rely solely on FA stores in blubber for milk fat production, and thus the milk of these animals has a FA composition most similar to that of the adipose tissue from which it was mobilized (*e.g.*, Iverson *et al.* 1995, Brown *et al.* 1999). As such, milk FA will be indicative of diet over the months of fattening prior to lactation. Conversely, in income breeders (*e.g.*, otariids, small odontocetes), milk FA will reflect the most recent dietary intake (*i.e.*, days), as well as some mobilization of FA from fat depots. Hence, the information that milk FA can provide about diet must be considered within the context of a species' lactation strategy. Although the direct influence of diet and blubber stores on milk FA are clearly evident (*e.g.*, Wamberg *et al.* 1992, Iverson 1993, Iverson *et al.* 1995), quantitative diet estimates using milk FA and QFASA have not yet been attempted or validated.

At the shortest time scale, lipids can also be isolated from blood in the form of chylomicrons. Chylomicrons only persist in the blood for a few (~2–6) hours (Cooper *et al.* 2005). When properly isolated from blood, experimental evidence from gray seals has shown that chylomicron FA can provide accurate estimates of the most recent meal (Cooper *et al.* 2005).

Tissue Sampling

Blubber and adipose tissue collection—As stated above, metabolic adipose tissue, such as subcutaneous and mysenteric fat or the energy storage area of blubber, must be sampled to make inferences about diet. Thus, an important consideration is the specific location on the animal from which the sample is obtained. The distribution of fat within and over the body can be heterogeneous with respect to both morphology and lipid composition (Ryg *et al.* 1988; Doidge 1990; Pond *et al.* 1992, 1995; Koopman 1998; Iverson 2002); this is species-specific. Fat tissue sampled on any extremity, such as limbs or fins, will likely be more structural in nature and should not be used without validation studies. In contrast, the FA composition of subcutaneous adipose tissue sampled over the main part of the body appears to be relatively homogeneous in many species. In seabirds, mink (*Mustela vison*), and polar bears (*Ursus maritimus*), samples collected from several subcutaneous or mysenteric adipose tissue sites within an individual do not differ significantly (Layton *et al.* 2000, Iverson and Springer 2002, Thiemann *et al.* 2006). Although it will be valuable to confirm the uniformity in FA composition of adipose tissue depots with other species, it appears that the best site for sampling subcutaneous adipose tissue is simply where most fat is normally

stored, which will largely be species-specific. Evidence from gray seals suggests that blubber lipid composition is quite uniform throughout the main trunk of the body (*i.e.*, dorsal, ventral, lateral, anterior, and posterior) in phocid seals (Cooper 2004). Nevertheless, to minimize sources of variability unrelated to diet, we recommend that samples be collected from the same location on the animal; in phocids an ideal spot is the midflank where the blubber and fat storage is thickest (Ryg *et al*. 1988). In contrast, in otariids, the thickest part of the blubber, and site of greatest fat storage, may be the neck. In cetaceans, it has also been shown that the lipid composition of blubber does not vary significantly over the thorax region of the animal, but this is not likely the case for the structural blubber comprising the tailstock, as demonstrated in harbor porpoises (*Phocoena phocoena)*, long-finned pilot whales (*Globicephala melas*), common dolphins (*Delphinus delphis*), bottlenose dolphins (*Tursiops truncatus*), and Sowerby's beaked whales (*Mesoplodon bidens*) (Koopman *et al*. 1996, Koopman 2001).

An issue of perhaps greater importance is the depth of the tissue sampled. In polar bears, the FA composition of the superficial adipose layer appears to be highly uniform with depth (Thiemann *et. al* 2006). However, the FA composition of blubber has been shown to vary with tissue depth in marine mammal species that store blubber (West *et al*. 1979, Käkelä and Hyvärinen 1996, Koopman *et al*. 1996, Koopman 2001, Best *et al*. 2003, Thiemann *et al*. 2004). In pinnipeds, blubber near the skin surface appears to be more structural in nature, containing higher concentrations of endogenous FA and likely experiencing somewhat lower turnover rates. Conversely, blubber FA nearer the body core are more metabolically active and turn over more rapidly (Cooper 2004). Nevertheless, as stated previously, studies using radio-labeled FA demonstrate that the deposition of labeled FA into the outer layer of pinniped blubber occurs within 12 h of ingestion, although a higher concentration is found closer to the body core (Budge *et al*. 2004). This study, as well as other controlled feeding studies, shows that the full-depth blubber layer provides information on longer-term diet while the inner half alone reflects more recent diet (Iverson *et al*. 2004, Cooper 2004). The blubber of cetaceans, particularly odontocetes, generally exhibits more pronounced stratification than pinnipeds, but the degree to which this occurs is highly species-specific (Koopman 2001). In some cases, FA stratification is so extreme that only the innermost layer can be used to infer diet, as the FA stored in the outer layers seem to be largely independent of diet. For example, concentrations of major dietary FA, including PUFA, in the outer blubber layer of harbor porpoises are extremely low, and show little variation among animals from broadly distinct geographic regions (Fig. 3). Instead, this outer layer is comprised largely of FA arising from *de novo* biosynthesis. In contrast, levels of dietary FA, including PUFA, in the inner blubber layer are much greater (>4-fold) and more variable among regions (Fig. 3). This indicates that porpoises sampled from different geographic locations have different diets (*e.g.*, see Recchia and Read 1989, Aarefjor *et al*. 1995, Santos and Pierce 2003), a conclusion that would not have been apparent from analysis of only the outer blubber layer. However, stratification appears to be less pronounced in larger cetacean species such as the bottlenose whale (*Hyperoodon ampullatus*, Hooker *et al*. 2001) and sperm whale (Koopman 2001). Because stratification can vary within and among species, it is important to select tissues and sampling locations that are appropriate to address the research questions. Thus, it is important to sample the entire depth of an adipose depot and establish the degree of stratification to ensure recovery of the most metabolically active tissue and accurate inference of diet from FA analysis.

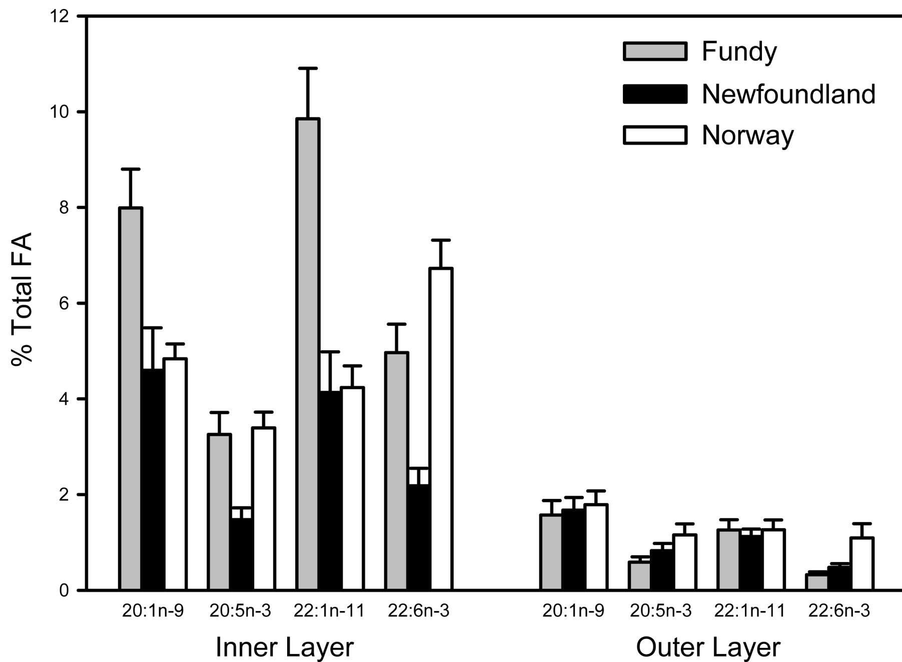


Figure 3. Concentrations (wt % ± SE) of selected FA of dietary origin in the inner (deep) and outer (superficial) layers of the blubber of male harbor porpoises (>125 cm in length) from the Bay of Fundy (n = 13), Newfoundland (n = 10), and Norway (n = 16).

Milk and blood collection—Although concerns over sampling location are not relevant with milk and blood samples, the time frame over which samples are collected is important. In a capital breeder, milk FA over the entire lactation period will reflect those mobilized from blubber stored prior to parturition. In contrast, in income breeders such as otariids, small odontocetes and small phocids that feed during lactation (*e.g.*, the harbor seal [*Phoca vitulina*], Bowen *et al*. 2001), milk FA will either reflect those mobilized from body stores, those consumed on the most recent foraging trip or both. For example, otariids and the harbor seal have a perinatal fast during which FA in milk are derived solely from blubber, whereas later in lactation, milk FA will generally be derived from feeding and storage, but the relative importance will depend on the fraction of total requirements that are derived from each FA source. Similarly, most odontocetes are too small to store large quantities of lipid, and must feed frequently during lactation, while mysticetes generally rely on lipid stored in blubber during the feeding season (Oftedal 1997). Thus, the interpretation of milk FA data must consider the physiological consequences of a species' lactation strategy.

If milk FA composition is of interest, a potentially convenient sampling strategy for animals, such as pinnipeds, is the use of stomach contents evacuated by intubation from offspring that have recently suckled. Stomach contents cannot be used to infer milk fat content or proximate composition due to rapid dilution by gastric juices, digestion, and passage (Iverson 1988). However, milk fat is secreted in globules so that the FA hydrolyzed in the stomach by gastric lipases remain with the parent globule

until reaching the intestine. Thus, despite extensive hydrolysis in the stomachs of suckling pinnipeds (Iverson *et al.* 1992), the FA composition of gastric contents remains identical to that of the milk consumed for up to 8 h following ingestion or as long as milk remains are apparent, as has been demonstrated in pups of harp seals, (*Pagophilus groenlandicus*), hooded seals (*Cystophora cristata*) and California sea lions (*Zalophus californianus*; Iverson 1988).

The time of collection with respect to feeding is a more important issue in the sampling of blood to examine dietary lipids, because of the ephemeral nature of lipid transport. It is the TAG-rich chylomicrons which carry the FA recently ingested by a predator that one must analyze. Therefore, it is essential that chylomicrons are: (1) visibly present in samples (as indicated by a cloudy or milky hue) and (2) isolated from other blood lipids and lipoproteins in order to infer diet. Only the FA of the whole chylomicrons, or ideally, the TAG isolated from these chylomicrons, can be analyzed to infer diet. The FA composition of the PL of blood lipoproteins, including chylomicrons, is highly conserved and contains little information about diet. If whole blood, plasma or serum are analyzed, the increased input of FA mobilized from endogenous stores, carried in the other lipid and lipoprotein classes, will lead to highly erroneous results in diet inference (Cooper *et al.* 2005). The isolation of chylomicrons from other lipoprotein classes is best done by ultracentrifugation of freshly collected serum or plasma samples (both give identical results), as even short-term freezing may cause disruption of the chylomicron lipids. In gray seals, it has been demonstrated that the FA composition of whole chylomicrons obtained from blood samples were most abundant and most accurately estimated diet 3 h after feeding (Cooper *et al.* 2005). The FA composition of whole chylomicrons sampled at greater time periods post-feeding less accurately estimated diet, but it is likely that separating the TAG from the PL in these latter chylomicrons would improve estimates. Meal size may also affect the optimum window for the recovery of chylomicrons (Cooper *et al.* 2005) and this is an area where further research is needed.

Prey sampling—To quantitatively estimate diet from the analysis of the fat stores of a marine predator, the FA composition of the potential prey must be known. In most cases, the prey are fish and invertebrates, although prey could also include mammals or seabirds. Issues concerning the analysis and development of these prey databases have been discussed elsewhere (Budge *et al.* 2002; Iverson *et al.* 2002, 2004). However, to summarize, the onus is on the investigator to reasonably sample the prey field of the predator and to sample it in the same manner in which the predator consumes these prey. Because most marine mammals and seabirds consume their prey whole, whole prey should be homogenized and analyzed. Although the inclusion of prey stomach contents may increase within-species FA variability, this is what must be characterized, as it is what the predator eats. Regardless, between-species differences in FA signatures are more pronounced than within-species differences. Sources of such within-species variability include prey size (*i.e.*, changes in diet with size) and major geographic location (*e.g.*, Budge *et al.* 2002, Iverson *et al.* 2002). Annual variation may also require investigation, although current evidence still suggests that this is minor compared to overall between species differences (*e.g.*, Iverson *et al.* 2002). Nevertheless, it is important to sample prey sufficiently to capture such variability so that the investigator can be confident that prey species can be reliably distinguished on the basis of their FA signature.

Finally, regardless of the tissue or species sampled, freshness is of utmost importance. Ideally, all tissue samples should be taken from live or freshly dead animals

(Smithsonian Institution codes 1 and 2; Geraci and Lounsbury 1993). Immediately after death, lipolytic enzymes begin to degrade FA resulting in a loss of PUFA (Christie 1982, Shaikh 1986). However, providing the specimen is freshly frozen and intact, lipolytic enzyme activity should be minimal. Homogenized samples degrade much more quickly as more tissue area is exposed to oxygen and thus require more careful storage (see below).

Sample Storage

Inevitably, some period of time elapses between sample collection and analysis, making storage of samples unavoidable. Exposure to air will result in oxidation of FA in the sample with a resulting loss of information. Freezing at −80°C in an airtight container is one recommendation for long-term storage (Shaikh 1986). However, this may not be possible in the field or in many labs. An equally reliable, if not better, alternative is to immediately preserve the lipids in the sample by immersing the sample in chloroform ($CHCl_3$) containing 0.01% butylated hydroxytoluene (BHT) to prevent oxidation. If frozen at −20°C, especially under a nitrogen atmosphere, such samples may be safely stored for periods of years. In this form, refrigeration or room temperature storage of such samples can be safely done for several weeks or months. However, it is critical that only glass vials with Teflon-lined caps be used for such storage, as $CHCl_3$ will extract the plasticizers in other types of containers and contaminate the isolated lipids. If storage in $CHCl_3$ is not possible, as is often the case for large fish or invertebrates, samples may be temporarily frozen in airtight containers at −20°C for months, but the probability of lipid oxidation increases in smaller samples with greater surface areas, and with the length of time elapsed before immersion in $CHCl_3$. Oxidation certainly will have taken place in samples that have been desiccated prior to determination of proximate composition, so accurate analyses of fat content and FA composition cannot then be carried out on such samples. Another option is to homogenize the prey and immediately place a weighed aliquot in $CHCl_3$ with BHT, for extraction at a later date.

Guidelines for the optimal sampling and storage of tissues for lipid analysis are summarized in Table 1. Recognizing that these procedures are not always practical or possible in the field, recommendations for temporary storage when solvents or refrigeration at −20°C may not be available are also provided. We stress, however, that lipid content and FA composition of samples may be compromised in these situations, and these procedures should only be used when no other alternatives are available.

Blubber and adipose tissue—Immediately after collection, small samples (<2 g), such as biopsies (<0.5 g), should be sealed in aluminum foil, taking care to not crush the tissue and to keep it cool. If the sample is a blubber core that has been subsampled, each subsample should be separately sealed in foil and labeled. Upon returning to the lab (*i.e.*, within the day of collection), any fragments of skin or muscle should be removed from the lipid sample, which should then be weighed and placed in a small volume (*e.g.*, ~2 mL for ≤ 0.5 g sample) of $CHCl_3$ containing 0.01% BHT to prevent oxidation. Alternatively, if the sample is taken from a dead animal, larger samples can be placed in a sealed plastic bag and frozen. Prior to extraction, the exterior margins of the tissue should be trimmed, removing oxidized tissue.

Milk and blood—Milk should be treated in the same manner as blubber: either frozen immediately for limited times in airtight containers or, better, aliquoted into

Table 1. Recommended sample storage practices for lipid analysis.[a]

Sample type	Recommended storage	Duration	Temporary storage	Duration
Blubber	In $CHCl_3$ containing 0.01% BHT at −20°C	Years	Large pieces (>0.5 g) may placed in airtight container and stored at −20°C.	Weeks (if piece is >0.5 kg, can store for ≥year and take core from center for analysis)
Milk	Aliquot in $CHCl_3$ containing 0.01% BHT at −20°C	Years	In airtight containers at −20°C	Weeks to months
Blood	NA		Centrifuge to isolate plasma or serum and refrigerate	Days
Isolated blood chylomicrons	Store in $CHCl_3$ containing 0.01% BHT at −20°C	Years	NA	
Fish and large invertebrates	Aliquot of homogenized whole individual in $CHCl_3$ containing 0.01% BHT at −20°C[b]	Years	Whole individuals in plastic bag or other airtight container at −20°C	Months to ∼year
Small invertebrates	Filter to remove water, store whole in $CHCl_3$ containing 0.01% BHT at −20°C[b]	Years	Filter to remove water, store whole individuals in plastic bag or other airtight container at −20°C	Weeks to months
Terrestrial plants	In isopropyl alcohol with BHT added at −20°C	Years	Refrigeration (4°C)	1 wk

[a]Although airtight storage at −80°C is reliable for long-term storage (years), we have recommended here the most prudent form and lengths of storage, while trying to build in some flexibility for the investigator. However, it should be understood that during all types of temporary storage listed, oxidation will begin to occur and damage to FA structure will increase with length of time. When storing in $CHCl_3$, solvent-rinsed (with $CHCl_3$ or methylene chloride) glass containers with Teflon caps must be used. Caps should be tightened and then anchored in place with parafilm, plastic or Teflon wrap, wrapped in the direction of tightening, and containers stored upright. Plastic or parafilm must not come into contact with the $CHCl_3$ itself. If possible, containers should also be topped with a stream of nitrogen for long-term storage. A ratio of about 10:1 $CHCl_3$ volume (mL): sample mass (g) should be used. For instance, for a typical 0.2–0.5 g blubber biopsy or milk sample, 2 mL of $CHCl_3$ are used. Depending on the size and fat content of the prey, the aliquot should be large enough to extract and accurately measure the lipid content. In all cases, storage in solvent with BHT (butylated hydroxytoluene, an antioxidant) will best preserve the sample. Please see text under "Lipid Extraction" for clarification on the use of $CHCl_3$ and BHT.

[b]If long-term storage of homogenized prey is necessary, a measured 1.5 g aliquot should be stored in a precisely known volume of $CHCl_3$ (*e.g.*, 4 mL). At extraction (see Appendix 1), instead of a preliminary addition of MeOH, the full volume of MeOH (10 mL) and the specified volume of $CHCl_3$ minus that already present (*i.e.*, 20 mL − 4 mL) should all be added at once and the rest of the procedures followed as described.

$CHCl_3$ with BHT and frozen. If samples cannot be frozen, they may be stored for days or weeks in $CHCl_3$ with BHT, but should be kept in the dark in as cool a place as possible.

Blood samples should be initially centrifuged at approximately 100 × *g* for 20 min to separate the lipid-containing plasma from the red blood cells. Ideally, chylomicrons would then be isolated and concentrated from the plasma by ultracentrifugation at this point. However, if necessary, plasma samples may be stored at 4°C in the dark for several days before separating the chylomicrons from other lipoproteins. Ethylenediaminetetraacetic acid (EDTA; 1 mg/mL of plasma), an antioxidant and anti-coagulant, must be added to the plasma before storage (Bachorik 1982). Chapman and Kane (1975) also suggest the addition of sodium azide (0.2 μg/mL), an anti-microbial agent. Appendix 2 describes a typical procedure for the separation and recovery of chylomicrons from plasma samples (Cooper *et al*. 2005).

Fish and large invertebrates—Fish and large invertebrates (*e.g.*, squids, lobsters) should be frozen whole in well-sealed plastic bags immediately following collection. Just prior to analysis, specimens should be thawed, and then homogenized. As stated above, if longer-term storage is required prior to analysis, fish can be homogenized and then aliquoted into $CHCl_3$ with BHT. Fish can generally be easily ground in a blender, and for invertebrates with exoskeletons, mallets can be used to crush the shells before homogenizing. Normally, lipids are extracted from a subsample (*ca.* 1.5 g) of the homogenized material. After homogenization, the sample is very susceptible to oxidation, so if a second subsample of ground material is to be archived for future use, it should be placed in a small plastic bag, sealed and immediately frozen at −80°C (or −20°C for limited periods).

Small invertebrates—Small crustaceans such as euphausiids and amphipods are important components of the diets of many seabirds (Bradstreet 1980, Hobson 1993), baleen whales (*e.g.*, gray whales *Eschrichtus robustus* [Nerini 1984] and bowhead whales *Balaena mysticetus* [Carroll *et al*. 1987, Lowry 1993]), and some seals (*e.g.*, ringed seal *Phoca largha,* harp seal, Antarctic fur seal *Arctocephalus gazella*, Weddell seal *Leptonychotes weddellii*, crabeater seal *Lobodon carcinophaga* [see Reeves *et al*. 2002]). These species are typically collected using trawls and must be gently filtered through an appropriately sized plastic mesh (typically <100 μm for marine mammal and seabird prey) to remove excess water before weighing and freezing in plastic bags or glass containers. Alternatively, if large enough, specimens can be removed individually from the water, weighed, and placed in $CHCl_3$. Individual samples should be pooled to obtain samples weighing 0.5–1.5 g. Small invertebrates are often stored in alcohol or formalin for later microscopic identification, but these preservatives make quantitative lipid extraction impossible. Therefore, a subsample should be taken for identification and the remainder frozen for lipid analysis.

Terrestrial plants—Plants contribute to the diet of some marine predators such as manatees and polar bears and, therefore, warrant consideration. Plants contain very active lipolytic enzymes and the action of these enzymes should be minimized by storing in and performing the initial lipid extraction with isopropyl alcohol (Christie 1989).

Chemical Methods

Lipid extraction—To determine the fat content and FA composition of predator adipose and potential prey species, including FA precursors (alcohols or DMA), lipids

must first be extracted from the sample. Although there are a variety of extraction methods in the literature (*i.e.*, Folch *et al.* 1957, Bligh and Dyer 1959, Smedes 1999), one of the most convenient and accurate procedures involves lipid extraction with a modified Folch *et al.* (1957) procedure employing $CHCl_3$ and methanol (MeOH). A common error is the application of the Bligh and Dyer (1959) extraction technique to samples high in fat. The Bligh and Dyer (1959) extraction was originally designed to extract lipid from lean fish samples ($\leq$2% fat) and will invariably underestimate lipid content in blubber and high fat fish such as herring and capelin (Iverson *et al.* 2001*a*). The modified Folch *et al.* (1957) procedure for extraction of prey is described in Appendix 1, and a somewhat simplified procedure for the extraction of adipose tissue, blubber, milk, and chylomicrons is given in Appendix 3. Both procedures can be scaled up or down as necessary as long as the ratio of solvent:water remains at 8:4:3 $CHCl_3$:MeOH:H_2O. $CHCl_3$ is a carcinogen and care must be exercised when used (*i.e.*, fumehoods must be employed). Some researchers have substituted the less toxic dichloromethane for $CHCl_3$ during extraction (Kattner *et al.* 2003, Böer *et al.* 2005) or have used other solvents or mixtures (Stenstrom *et al.* 1986, Cabrini *et al.* 1992, Undeland *et al.* 1998, Smedes 1999, Smedes and Askland 1999, Lin *et al.* 2004). However, none of these solvents, including dichloromethane, approach the full lipid recoveries obtained with $CHCl_3$ and MeOH. Thus, until further verification, this mixture continues to be recommended and used by most labs. Finally, we recommend the addition of BHT during the storage, extraction, and transesterification process, as it effectively prevents oxidation of unsaturated FA and does not interfere with the separation of lipid classes in the TLC solvent system we recommend for isolating fatty alcohols and DMA, nor in the GC analysis of FA (see Appendices 6 and 7).

Transesterification (formation of FA methyl esters)—Once extracted, acyl lipids are transesterified (*i.e.*, converted directly to FA methyl esters or FAME; for other esters, see below) using a basic or acidic catalyst. Typical basic catalysts include potassium hydroxide (KOH) and sodium methoxide; common acidic catalysts are boron trifluoride (BF_3), sulfuric acid (H_2SO_4), and hydrochloric acid (HCl). In situations where free FA may be present (*e.g.*, gastric milk samples, improperly stored milk samples, thawed fish samples that have been homogenized in a blender), basic catalysts should be avoided as free FA will not be methylated with this technique, producing inaccurate FA compositions.

Most acid catalysts seem to produce equivalent results. Recent studies have demonstrated that comparable results are obtained using fresh anhydrous BF_3 and H_2SO_4 catalysts (Iverson *et al.* 1997*a*, Thiemann *et al.* 2004). However, more recently, we and other labs have encountered problems with BF_3 that have resulted in incomplete transesterification, likely due to moisture in the reagent. Apparently, anhydrous BF_3 in MeOH is no longer guaranteed by chemical suppliers selling it. Incomplete transesterification can be detected by rapid degradation of the GC column, leading to inaccurate analyses, and the accumulation of organic deposits in the injection liner (see "GC components"). This is consistent with earlier observations that the use of old or poorly stored BF_3 catalyst may produce artifacts or spurious peaks in the chromatogram and result in loss of PUFA (Christie 1982). Although some groups continue to use BF_3 without apparent problems, we now recommend the use of the H_2SO_4 catalyst instead for creating FAME, which is also recommended by Christie (1982). A straightforward, reliable procedure for using the H_2SO_4 catalyst is summarized in Appendix 4. (Note: BF_3 in butanol, used for generating FA butyl esters [FABE; see "Samples containing unusual or problematic lipids"] does not seem to suffer from contamination with water and continues to be used reliably.)

In addition to the BF_3 issue, several other potential pitfalls are associated with transesterification techniques. First, as mentioned above, acid-catalyzed transesterification, regardless of the specific acid, is inhibited by the presence of water. Therefore, all lipid extracts must be treated with a drying agent (*e.g.*, anhydrous sodium sulfate, Na_2SO_4) prior to transesterification to ensure that all water is removed. All solvents must also be of high purity, and optima grades with impurities <1 ppm is the best choice; traces of water in low purity reagents can lead to problems in formation of volatile esters. (However, treating the BF_3/MeOH reagent with anhydrous Na_2SO_4 does not alleviate the problems encountered with its use.) Second, exposure of samples containing long-chain PUFA (commonly encountered in marine samples) to elevated temperatures in air may lead to oxidation of those FAME. To avoid this, transesterification reactions must take place in a nitrogen atmosphere, achieved simply by blowing nitrogen into the vial containing the sample and quickly capping it. Third, acid catalysts in MeOH have limited shelf lives. To avoid problems associated with aging, fresh reagents should always be used.

An alternative procedure for the formation of FAME, "direct" transesterification in which extraction and methylation are done in one step, has been employed in several studies (Grahl-Nielsen and Mjaavatten 1991, Liu 1994, Guillou *et al.* 1996, Cantellops *et al.* 1999). Typically, this procedure involves treatment of a very small (<25 mg) sample of blubber or fish with a solution of HCl in MeOH, brief heating and extraction of FAME with hexane. While it is a reliable method for transesterifying FA present (Thiemann *et al.* 2004), the potential problem lies in the extent to which a very small sample can be representative of the FA composition of the overall tissue. As described previously, blubber FA composition varies with depth of tissue. Thus, this micro technique cannot provide an accurate representation of the FA composition of the full depth or even a selected depth of the blubber core (Thiemann *et al.* 2004). However, if the sample is completely homogeneous (*e.g.*, milk or homogenized prey), or if a macro-modification of direct transesterification is made (to transesterify the full depth of tissue), then results are comparable to those using extraction and H_2SO_4 as described above (Thiemann *et al.* 2004). Thus, this direct method can be quite useful for analyzing the FA composition of very small whole tissues, such as the complete adipose biopsy from a small seabird, or of prey such as single zooplankton, which otherwise would be too small to analyze individually. However, researchers should be aware that direct transesterification cannot provide accurate estimates of the fat content of prey, which are needed in quantitative models to estimate diet composition.

In addition to methyl esters, nitrogen derivatives of FA, such as picolinyl esters, may be prepared to then analyze using mass spectrometry fragmentation patterns (*e.g.*, Wetzel and Reynolds 2004). However, the use of picolinyl esters has largely been developed for, and confined to, agriculture research because the number of FA structures to resolve is fairly small compared to that present in marine samples. There are serious problems to consider when using this type of analysis with marine samples (see "FA identification and data collection"), and thus it is not recommended for routine analysis.

Samples containing unusual or problematic lipids—Short-chain FA: Short-chain FA (those <14 carbon atoms in length) are present in considerable proportions in the adipose tissues of several members of the toothed whale families. Because almost all are formed *de novo* in the predator (see "FA modification and biosynthesis") and even if consumed in diet would be oxidized before deposition (see "Lipid and products"), these compounds are of little direct interest in diet studies. However, they can reach

quite significant concentrations in adipose tissue (*e.g.*, the blubber of belugas, *Delphinapterus leucas*, and Hector's dolphins, *Cephalorhynchus hectori*, can contain 10–17 wt% and 20–41 wt% of *i*-5:0, respectively; Koopman *et al*. 2003), and failing to quantify these short-chain compounds will result in the artificial inflation of the concentrations of all other FA of both endogenous and exogenous origin. Currently, the relationship between the presence of short-chain FA and the deposition of both endogenous and dietary lipids, and whether one influences the other, is unknown. Until this is better understood, it is prudent to determine the entire FA profile, including the short-chain compounds. Furthermore, the quantification of these short-chain, nondietary FA provides unique insights into other areas of physiological ecology (*e.g.*, Koopman 2001, Koopman *et al*. 2006), as well as the nature of the tissue being examined. For instance, tissues with high concentrations of short-chain endogenous FA tend to exhibit low turnover and thus will be less representative of diet (Koopman 2001, Koopman *et al*. 2003).

Short-chain FA cannot be analyzed using conventional FA techniques because of their high volatility and solubility in water. Methyl esters of short-chain FA are even more volatile than their FFA counterparts so large or complete losses of these FAME are inevitable, producing erroneous results. If short-chain FA are present in a sample, alternative methods must be used to accurately identify and quantify all FA. Although this may deter some researchers from giving short-chain lipids proper consideration, these compounds do contain important information and are readily quantified using minor modifications to conventional methods. Determining short-chain FA to begin with also avoids the potential later need for the investigator to perform a second analysis. The simple solution is to form a heavier, less volatile derivative by substituting a butyl group for the methyl group in the FAME, creating FA butyl esters (FABE) (Iverson and Sheppard 1977; Koopman *et al*. 1996, 2003; see Appendix 5). These heavier derivatives, while less volatile, are still subject to evaporative losses if warmed under a stream of nitrogen, so care must be exercised and evaporation steps avoided wherever possible. We have yet to encounter any problems of incomplete transesterification with the BF_3/butanol reagent used in this procedure.

Wax esters: A number of marine animals store fat as WE (see "Lipid structures"). WE are easily extracted along with TAG and other acyl lipids from marine samples with the Folch et al. (1957) method. Acid- or base-catalyzed transesterification of this extract will hydrolyze the WE, producing one FA methyl ester and one fatty alcohol per WE molecule (Fig. 2B). On a polar GC column (one with a 50% cyanopropylsiloxane phase; see "GC components"), fatty alcohols can be recognized by the presence of broad, poorly resolved peaks on which FAME may elute (Fig. 4). If a different phase is used (*e.g.*, FFAP-nitroterephthalic acid modified polyethylene glycol), alcohols with <22 carbon atoms can be identified as sharp peaks, but they often co-elute with FAME. Because of poor resolution and co-elution, alcohols cannot usually be analyzed on the same capillary column, during the same run, as FAME. A thin-layer chromatography (TLC) separation is first carried out to separate alcohols and volatile FA esters, and those two groups are then recovered individually from the TLC plate by solvent extraction. At this point, the alcohols can be modified to create a volatile derivative, usually an acetate or silyl derivative (requiring optimization of the GC, see "GC Analysis of FAME"; Zweig and Sherma 1972, Christie 1989), or analyzed directly on the appropriate column. Certainly, the presence of fatty alcohols complicates the analysis and requires additional time-consuming procedures. However, if diet estimation is the primary interest, it is essential to determine total

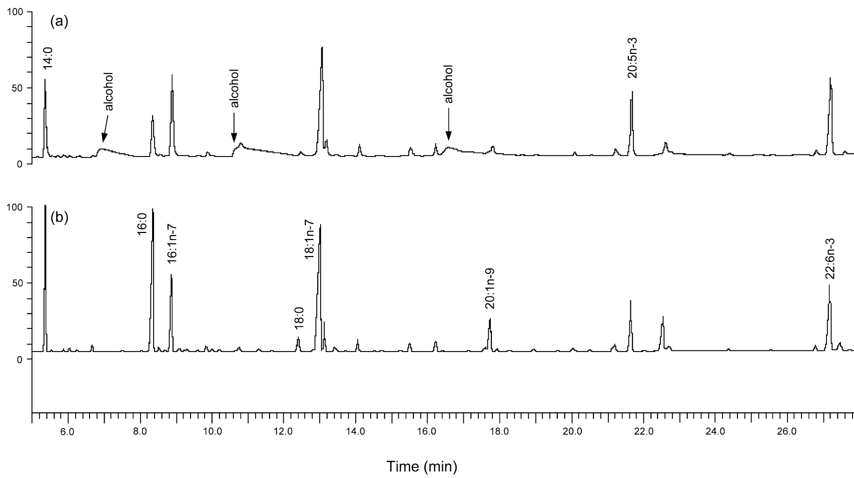


Figure 4. Selected portion of a chromatogram showing (a) alcohols among FAME peaks and (b) the change in FAME concentration after oxidation of alcohols.

amounts of both FAME and alcohols before GC analysis so that their proportions can later be recombined to produce an accurate FA signature (see "Lipid digestion and products").

As FA and fatty alcohols are metabolized similarly after digestion, a convenient analytical solution is to oxidize any alcohols to free FA, which can then be methylated with the transesterification procedure described previously. The method uses a modification of a standard oxidizing agent containing chromium oxide, known as Jones' Reagent (Bowden *et al.* 1946). Because the presence of alcohols is not normally recognized until the lipid extract has been transesterified and analyzed on the GC, the procedure begins with the initial product of transesterification, containing alcohols and FAME. These alcohols are first separated from FAME by TLC. They are then treated with oxidant and the resulting free FA are esterified with H_2SO_4/MeOH (Budge and Iverson 2003; Appendix 6). The initial separation of FA and alcohols is necessary because the oxidation reagent reacts with highly unsaturated compounds. Thus, if PUFA are not removed before adding the oxidizing reagent, their proportions will be reduced in the resulting mixture. Conveniently, fatty alcohols derived from WE are almost always saturated or monounsaturated (Sargent 1976) so that losses of the alcohol fraction due to the oxidation of double bonds are negligible.

Plasmalogens: Small amounts of plasmalogens are found throughout the animal kingdom and in certain anaerobic bacteria (Horrocks 1972). As such, it is surprising that they have not been detected more often in GC analysis. It is the vinyl-ether component of the plasmalogen that is problematic in FA analysis. The vinyl ether is hydrolyzed to the corresponding aldehyde when treated with an acid, such as BF_3 or H_2SO_4 in MeOH (Fig. 2C). However, this aldehyde, rather than being enzymatically oxidized to a FA as it is after ingestion, is chemically oxidized to form a DMA (Fig. 2C). On polar GC columns, DMA co-elute with specific FAME, depending on the column employed. For example, on a polar 50% cyanopropyl-methylpolysiloxane column, 16:0 and 18:0 DMA co-elute with 15:1n-6 and 16:4n-3 FAME, producing spurious results (Budge and Iverson 2003). On a less polar polyethylene glycol column, the

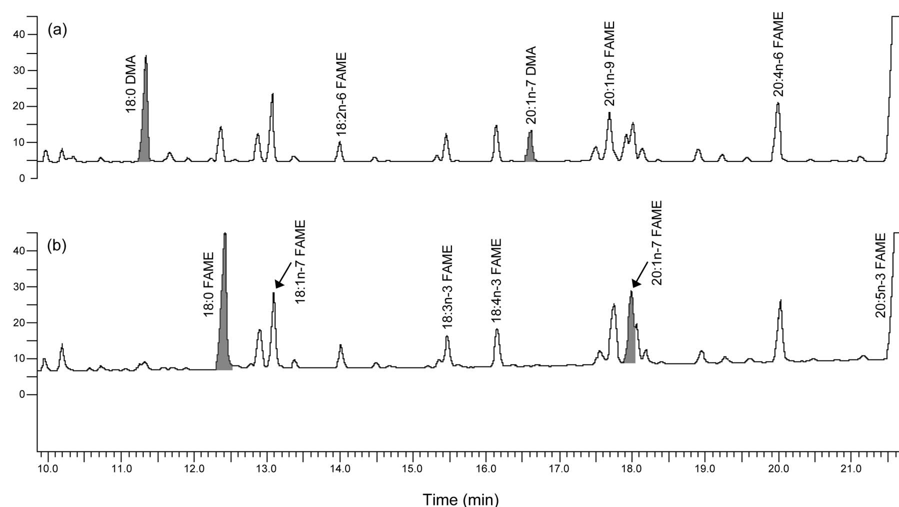


Figure 5. Selected portion of chromatogram showing (a) the presence of DMA among FAME peaks and (b) the change in FAME concentration after oxidation of DMA. Reproduced from Budge and Iverson (2003).

same two DMA co-elute with the branched chain FAME *i*-16:0 and *i*-18:0 (Ackman 1998, Budge *et al.* 2001). Typically, DMA peaks are slightly broader and are present in much greater amounts than could be biologically possible in the FAME peaks with which they co-elute, so the presence of DMA can often be determined through inspection of the GC chromatogram (see Fig. 5).

When DMA are recognized in a FAME chromatogram, one of two courses of action can be taken. If only the acyl composition is required, the original lipid extract can be transesterified again using a basic catalyst that does not cleave the vinyl-ether linkage so that DMA are not produced (Christie 1982). However, if diet estimation is the goal, the proportions of all FA precursors, including DMA, in the prey are required. Because DMA are normally limited to saturated or monounsaturated structures (Horrocks 1972), the same oxidation procedure described for alcohols can be applied (Appendix 6). As with the alcohol analysis, DMA must first be separated from FAME using TLC. For this separation, we use 100% toluene as the developing solvent. If alcohols are present in a sample with DMA, toluene may also be employed, with the alcohols eluting just below the DMA, near the origin. In that situation, the alcohol and DMA portions may be removed from the TLC plate and combined for oxidation.

GC Analysis of FAME

GC components—For routine FA determination, GC is invariably used (*e.g.*, Ackman 1986, Christie 1989). A mixture of FAME in hexane is introduced into a heated injector, which causes the solvent and FAME to enter the gas phase. The solvent and FAME are then carried by a stream of inert gas (often helium or hydrogen) through a column coated with a liquid in which the FAME are retained to varying degrees depending on chain length and number and position of double bonds. This selective retention results in the FAME eluting individually at the end of the column, where they are measured by a detector, usually a flame ionization detector (FID).

The arrangement of components in GC can be quite complex but, in a basic setup for FAME analysis, the necessary parts include an autosampler, an injector, an oven containing a capillary column, a detector, and a computer interface. The injector is a heated chamber where the sample enters the gas phase. We have had best results with a split injector, where a portion of a concentrated sample is swept away by a stream of gas before entering the column. The remaining portion is then carried into the column that is contained in an oven. For particularly dilute samples (~0.5 mg/mL), splitless injection may also be employed, which is useful when the amount of FAME recovered from a sample is very small. The temperature of the oven is programmed to rise during the analysis for optimal resolution of all FAME in a reasonable time (see Iverson *et al*. 1997*b*, for example). Depending on the column coating, the FAME mostly elute from the column one by one and are detected by FID. When organic compounds such as FAME enter the flame of the FID, they are ionized and produce an electrical current that is amplified and measured. Other detectors, such as the mass spectrometer, are useful for confirmation of FA structures, but are not necessary for routine FA determination once identifications have been verified. The performance of each component in this basic system must be optimized to ensure adequate resolution of all FAME peaks (Grob 1995, Rood 1995). However, there are some important issues concerning GC analysis of FA for diet estimation that merit particular attention, and these are outlined in the following section.

Column selection—Capillary columns with different liquid phases suitable for a variety of applications are available from commercial manufacturers. For FA analysis, it is crucial to select a column that allows adequate separation of all peaks of interest. For instance, it is necessary to separate certain critical pairs of FAME that are particularly useful in marine prey differentiation, such as the many isomers of long-chain PUFA and some MUFA (*e.g.*, n-11 and n-9 isomers of 20:1 and 22:1). This can only be accomplished through the use of a polar capillary column. Excellent separations are achieved using a very polar column coated with (50% cyanopropyl)-methylpolysiloxane, specifically the DB-23 model from Agilent Technologies. However, other investigators have achieved good FAME separation with polyglycol phases, such as DB-WAX (Ackman 1986). Individual FAME analyzed on different columns will, of course, have different elution/retention times and orders. The best practice is to find a polar column that produces satisfactory results and use it consistently. Non-polar columns are not appropriate for analysis of marine FA because of co-elution of peaks and the inability to resolve many important FA and isomers.

Although the best results are achieved using the most polar columns, these columns are less stable and more susceptible to degradation. Column performance degrades over time due to loss of the liquid phase with high temperature and repeated sample injection, and this degradation can be recognized by a decrease in peak resolution and shape (broadening and tailing). It is often possible to restore column performance by removing approximately a meter of the column that is closest to the injector and re-installing the column ("clipping" the column). It is often recommended by many companies to "solvent wash" or flush a polar solvent, such as dichloromethane, through the column to remove any non-volatile components that may have deposited. However, this results in a loss of the liquid phase, which changes retention times and sometimes results in poorer separations, especially with butyl esters. Thus, we do not recommend this technique. Eventually, with any column, performance will degrade to such an extent that the column must be replaced. It is important to do this earlier rather than later, given the critical nature of accurate FA quantification for diet estimation. Although a single column may cost about US$500.00, if 500

samples are run in duplicate this equates to only $1.00 per sample, a small price to pay for one of the most important steps in the analysis. If samples are clean, without contaminants and with complete transesterification, one can readily achieve 3,000–4,000 GC runs for each DB-23 column. It is important to recognize that replacement columns that manufacturers claim to be identical may not always produce identical results. In addition, columns with the same chemical phase but produced by different companies may produce widely different peak retention times and peak resolution. Thus, care must be taken to correctly re-identify each peak with any change in column or phase. A specific problem relevant to the DB-23 column is that of the resolution of 22:6n-3 and the much smaller 24:1. These two peaks may be easily separated on one DB-23 column, but may almost co-elute on another. This co-elution problem can usually be corrected by adjusting the temperature program, baking (conditioning) the column for an hour at its maximum temperature, or by increasing the carrier gas flow rate. The operating parameters that we routinely employ for GC-FID analysis of FAME and FABE are given in Appendices 7 and 8.

Polar columns are also very sensitive to oxygen at high temperatures. To avoid damage to the column, there must always be a flow of carrier gas through the column even when the GC is not in use. The carrier gas line must also be fitted with an oxygen trap to remove traces of oxygen from the gas and all fittings should be checked periodically for leaks. A rising baseline (or "column bleed") is indicative of oxygen damage. Such damage is permanent and results in poor peak separation. Column life may also be prolonged by the use of a guard column. A guard column is a segment of uncoated capillary tubing (1 m) that is placed between the injector and analytical column. The guard column collects any non-volatile components that may otherwise be deposited at the beginning of the analytical column and reduce column performance. Routine maintenance, such as periodic cleaning of the glass injector liner and replacement of septa, will also prolong the life of the GC column and preserve peak resolution. Regularly cleaning and repacking the injector liner with glass wool after 300 injections, and replacing the septum that the syringe needle pierces after 75 injections will greatly increase the lifespan of the column and quality of analyses.

FA identification and data collection—GC peaks corresponding to individual FA are identified and integrated (*i.e.*, quantified) using manufacturers' software. However, blindly accepting the FAME data generated by the computer software is a dangerous practice. A slight shift in retention time will result in incorrect identifications of peaks by the software. Misidentification by the software can also occur if concentrations of dominant FA are highly variable within a group of samples. In addition, the software programs use algorithms to establish baselines, separate peaks in close proximity and cut shoulders off large peaks. Occasionally these algorithms can produce unusual and incorrect peak shapes, resulting in large errors in peak areas. Because of these potential problems, each chromatogram must be checked manually for correct peak identifications and baseline manipulations, using software that will allow reintegration. Such inspection will also alert the investigator to irregularities in chromatography, such as a change in retention time that may be indicative of column degradation. This process of chromatogram inspection is essential if one wishes to use FA to quantitatively estimate diet, as even small errors in FA identification or quantification can cause large errors in diet estimation.

Before the software can identify peaks in the chromatogram, and ideally before any samples are run through the GC, all FA components must be initially identified by the analyst. One approach is to buy verified FA standards from chemical

manufactures, such as Sigma-Aldrich, Nu-Chek Prep, or Matreya. Such standards will contain known proportions of specific FAME that aid in both identification and verifying quantification; however, verified standards do not exist for all marine FAME. Therefore, it is generally necessary, in conjunction with the use of pure chemical standards, to purchase well-characterized natural FA mixtures such as menhaden oil. These mixtures contain practically all FAME that one could expect to encounter in a typical marine sample. The identities of peaks in such mixtures are sometimes pre-determined by the manufacturer or can be determined using published chromatograms such as those in Ackman (1986) and Ackman (1994). Particularly useful is Ackman's (1986) review in which peak identities in canola oil are compared to those in a fish oil, designated PUFA-1, and available from Matreya. Additional confirmation of peak identities is possible using equivalent chain lengths (detailed in Christie 1989) and especially mass spectrometry (MS; *e.g.*, Christie 1998). While MS is far more expensive than GC analysis, investigators can normally contract a lab to analyze several representative samples or sample types, from which all peak identifications can be confirmed. FA retention times are consistent. Therefore, once the elution pattern and, hence, identities of FA have been determined with a particular column, subsequent identification in different sample types using the same column is reliably done by retention time. Thus, it is not necessary to use MS for routine FAME identification. Likewise, analysis of MS fragmentation patterns of nitrogen derivatives of FA, such as picolinyl esters (*e.g.*, Wetzel and Reynolds 2004), can be very useful in originally identifying unknown FA, but it is certainly not necessary to employ these, or similar FA derivatives, each time a sample is analyzed for FA content. There are also serious shortcomings in the use of picolinyl esters. Picolinyl esters have boiling points about 50°C higher than those of the equivalent FAME (Christie 1998), which means that these compounds will only elute at temperatures far above the upper limits of polar columns. Thus, relatively non-polar stationary phases must be used and on such columns, adequate resolution of FA isomers is impossible (see "Column selection"). Certainly, the separation of 16:1, 18:1, 20:1, and 22:1 isomers and the corresponding SFA that are critical in differentiating among prey species will not be achieved. This inadequate separation will result in MS fragmentation patterns that cannot be interpreted because they will be derived from several co-eluting peaks. More importantly, such co-elution will prevent accurate quantification of the FA, as illustrated very clearly by Christie *et al*. (1986). More recently, Christie (1998) suggested that a slightly more polar column, a Supelcowax 10, may be used to achieve better separation of picolinyl esters but co-elution of isomers with 20 or more carbon atoms remains a problem. To put simply, resolution of some 70 FA structures that are present in marine samples is not an easy task with a very polar column; it is an impossible task without such a column.

Finally, corrections for sample loss within the GC and, specifically, due to the use of an FID, must also be made. Carbon atoms are ionized to generate the signal in an FID and carbons with few or no hydrogen atoms, such as those associated with the acid functionality or with double bonds, are ionized less or not at all during combustion. Thus, FA with double bonds will produce a smaller signal than an equivalent amount of SFA of the same length. The contribution lost due to the carbon atom associated with the acid functionality will also become more important as chain length decreases. Small losses of FAME within the injector and on the column, combined with the signal loss with the FID, necessitate the use of "response factors". These are factors that are multiplied by raw peak area to generate corrected areas (*i.e.*, quantitative amounts of FA). They are determined by analyzing standards of

known amounts and, once calculated, should not need adjustment. Response factors can also be derived from chemical theory, based on number of carbon atoms in the chain and number of double bonds present; a list of these for most FA can be found in Christie (1989). These theoretical response factors are surprisingly close to those measured. An efficient way to determine the value of the actual response factors is to first assign the theoretical response factors for all FA identified in a chromatogram and then to run a series of extremely fresh quantitative standard mixtures. Most FA should be accurately quantified and those whose values are slightly awry can have their response factors changed in an iterative process until the most accurate overall values are obtained for the FA mixtures. Because the response factor is the same for a given carbon chain length and number of double bonds, it does not matter whether all isomers are present in a standard mixture.

When FA have been identified and peak areas corrected, individual FA are normally reported as percent of total FA, which is equivalent to the mass percent of total FA. These can also readily be converted to mol percent, using the molecular weight of each compound. However, analysts accustomed to working with other types of compounds, such as pollutants, where an absolute concentration is required, may prefer to add an internal standard. This is a compound of known amount whose peak area can be compared to all others to determine their concentration. If an internal standard is to be used, care must be taken to select a molecule that is not present in the sample and will not co-elute with any of the FA of interest. Typically, 19:0, 21:0, or 23:0 is used, depending on the sample composition.

Interpretation of FA Data

Using Predator FA to Qualitatively Infer Diet

A number of techniques exist to examine predator FA as trophic indicators. Because marine lipids contain over 70 different FA, multivariate statistical techniques are generally needed to best use the information contained in the data. Nonetheless, it remains an extremely useful practice to use bivariate techniques to visually inspect and understand differences among samples in levels of specific FA of interest (*e.g.*, as in Fig. 3). However, it is important to avoid common pitfalls such as performing multiple ANOVAs without adjustment (such as Bonferonni corrections) for multiple comparisons. Additionally, simply finding a significant difference in levels of a given FA between two groups does not indicate whether this difference is biologically meaningful (*i.e.*, a very minor difference in levels of a FA, but low variability within groups, can result in a significant *P* value) or whether the overall pattern of all FA differs between groups. In contrast, multivariate analyses allow comparisons among groups of the overall pattern of FA or subsets of FA. Nevertheless, low within-group variability can still result in a significant result when differences in overall levels of FA are quite small. Thus, it is always necessary to consider both the statistical and biological significance of such analyses.

Multivariate analyses have been effectively used to examine trophic interactions and spatial and temporal differences among groups of animals (*e.g.*, Rouvinen and Kiiskinen 1989, Iverson *et al.* 1997*b*, Dahl *et al.* 2000, Mayzaud *et al.* 2000, Walton *et al.* 2000, Iverson *et al.* 2001*b*, Beck *et al.* 2005). However, it is important to understand that such analyses alone usually reveal relatively little about the actual species composition of the diet. In only rare instances may a unique biomarker indicate

that a certain prey must occur in the diet of a predator. Occasionally, it may also be possible to suggest types of species that account for most of the diet if the system or likely prey choices are relatively simple or if FA signatures are particularly distinctive (*e.g.*, Iverson *et al.* 1997, 2001*b*), but, in principle, inferring diets directly from one or a few FA may be a risky practice. For instance, high levels of particular long-chain MUFA (20:1n-9 and 22:1n-11) are commonly used to indicate carnivorous feeding on copepods (reviewed in Dalsgaard *et al.* 2003). However, copepods could be heavily consumed by a higher trophic level prey, such as a herring, which is, in turn, consumed by an apex predator, imparting equally high levels of 20:1n-9 and 22:1n-11. Thus, in general, it is not possible to distinguish between direct consumption by the predator of copepods or herring using only one or two FA. In a second example, 14:1n-5 and 16:1n-7, which are generally higher in blubber than in fish, have been used to infer consumption of marine mammals by killer whales containing high levels of these FA in their blubber (Herman *et al.* 2005). However, 14:1n-5 is most commonly present in marine mammal blubber (even fetal blubber) *via de novo* biosynthesis in the mammal (Iverson *et al.* 1995), with little dietary contribution; thus it is currently not possible to know the source of 14:1n-5 in whales from just the examination of this FA alone. Likewise, 16:1n-7 is also a common product of biosynthesis in marine mammal blubber (Iverson *et al.* 1995, Budge *et al.* 2004) and it is difficult to evaluate its contribution from biosynthesis or prey in whale blubber without evaluating the full suite of FA in both predator and prey. Finally, visual inspection of the FA in a few prey species in comparison to the predator's FA composition, using either bivariate or multivariate techniques, cannot generally allow even qualitative estimates of prey importance in diets as it does not take into account predator metabolism and its very significant effects on FA deposition, nor the ways in which a complex mixture of prey signatures translates into the FA composition of the predator (Iverson *et al.* 2004). To provide estimates of the proportion of prey species in the diet, a quantitative model, such as that recently developed by Iverson *et al.* (2004), is required (see below).

Statistical analyses—Classification and regression trees (CART) have been used to study changes in diet and spatial scales of foraging in several pinnipeds (*e.g.*, Iverson *et al.* 1997*a*, *b*; Smith *et al.* 1997). CART divides the samples into a series of sequential dichotomous groups based on individual FA with the greatest deviances. CART is a non-parametric method that has the advantage of requiring fewer assumptions of the data than most other multivariate methods. For example, homogeneous covariance matrices are not required, the number of variables is not limited by sample size and variables need not be normally distributed, so that untransformed percentage data may be analyzed with this method. However, classifications made by CART are based on relative concentrations of FA, regardless of their dietary origin or their metabolism. Therefore, results of CART analysis should be checked to make sure that the FA upon which splits have been made are biologically meaningful. More importantly, CART provides no indication of overall relationships of FA compositions among samples.

Hierarchical clustering has also been used to examine FA patterns in zooplankton and euphausiids, as well as other prey types (*e.g.*, Najdek *et al.* 1994, Mayzaud *et al.* 2000, Iverson *et al.* 2004). This type of analysis is exploratory, and attempts to determine relatively homogeneous groups of individuals based on their FA composition. The results are displayed in a dendrogram where the distance between groups is a measure of their similarity. As in the case of CART, sample size is not restricted by numbers of variables and multivariate normality is not required. However, at best, clustering only highlights the species that are more similar or different than others based on average FA composition, and which FA are most important in

making those distinctions. It does not identify significant differences among groups of samples.

Multivariate analysis of variance (MANOVA) tests whether the mean differences among groups, based on a linear combination of response variables, could have occurred by chance. That is, it allows one to determine whether FA compositions vary significantly with group membership (*e.g.*, Kirsch *et al.* 2000, Budge *et al.* 2002). Implicit in the application of MANOVA and most multivariate techniques are several assumptions that are typically ignored. These are discussed in some detail in a number of texts (*e.g.*, Stevens 1986, Tabachnick and Fidell 1989) but, in summary, these three assumptions should be met: (1) the observations are independent, (2) the covariance matrices are homogeneous, and (3) the data are multivariate normal. Independence of observations is an issue that does not require special attention for FA data but should be addressed, rather, in the design of the experiment or test. However, the occurrence of heterogeneous covariance matrices may present problems with the analysis of FA data. One can minimize this problem by ensuring that the analysis includes more samples in a group than the number of FA examined. Even with this precaution, heterogeneous matrices will often result and the analyst will be left to determine their effects on the result. Finally, proportional FA data are never multivariate normal. The data must be transformed in some manner prior to statistical analysis. An arc-sine transformation is often used (Zar 1999), but this type of transformation was designed for binomial data. Instead, the following log transformation recommended by Aitchison (1986) for proportional data can be used: $x_{\mathrm{trans}} = \ln(x_i/c_r)$ where x_i is a given FA expressed as percent of total FA, x_{trans} is the transformed FA data and c_r is the percentage of a reference FA. We use 18:0 as the reference FA, but any FA that is thought to convey little information about diet can be used as the reference; 18:0 is usually a good choice and reliably quantified. This transformation often, but not always, produces normal data. Again, the analyst must assess the impact of deviations from normality on their results.

Two other techniques commonly used, discriminant function analysis and principal component analysis (PCA), also require the three assumptions outlined above. Discriminant analysis is used to classify samples into groups, and to describe differences among those groups, by creating a series of uncorrelated linear equations of the original FA variables. The scores of samples on several of those functions can be plotted to effectively reveal relationships among samples. The results of discriminant analysis are usually easy to interpret and the FA most responsible for the variation on each function can often be determined. Similar to discriminant analysis, PCA can describe relationships among variables but it can also reduce large numbers of variables to a few components that represent most of the variance in the data by combining correlated variables into new components. Vectors are computed for each sample that can be plotted as in discriminant analysis (*e.g.*, Dahl *et al.* 2000) or the new components can be submitted to other statistical tests (*e.g.*, Goedkoop *et al.* 2000). In fact, the use of PCA vectors in new statistical tests is a particularly useful application. For instance, rather than conducting MANOVA on a subset of FA, one could first carry out a PCA on a much larger FA data set (if sample size permits) and then use the PCA vectors in the MANOVA. In this way, one can be sure that practically all of the information contained in the FA data is included in the analysis, rather than only incorporating data from arbitrarily selected FA. Of paramount importance to both DFA and PCA, however, is an adequate sample size. Not only are more samples than variables (*i.e.*, FA) required, but it is recommended that a minimum of 20 samples per variable be used in discriminant analysis (Stevens 1986) and at least five samples per

variable in PCA (Stevens 1986, Tabachnick and Fidell 1989). Indeed, analyses with low sample-to-variable ratios are often not reliable or reproducible. Thus, if sample size is limiting, one must select fewer FA for use in a particular analysis, choosing FA that are expected to vary based on biological functions or, if no *a priori* hypotheses exist, simply have the greatest variability and abundance in the sample set.

Despite the emphasis placed on the statistical assumptions and requirements described above, it must be recognized that FA data sets rarely meet such criteria due to the proportional nature of the data. Data may be transformed repeatedly but may never be normally distributed. Likewise, covariance matrices may always be heterogeneous despite large sample sizes and it is important to be aware of these limitations and their potential effects. The ratio of sample size to variable number is one parameter that can be easily controlled and common sense should be used in all situations. No matter what test is used, the measurement of only a few samples on a large number of FA will not allow any estimate of variance and cannot be expected to produce reliable results.

Quantitative Estimation of Predator Diets Using FA Signatures

The greatest interest in FA signatures is their potential application in the actual estimation of predator diets. Although the statistical techniques described above address important questions about patterns and differences in FA compositions among individuals and populations, they cannot be used to determine the species composition of the diet. Given the complexity in the number and patterns of FA, both within a predator and among potential prey species and individuals of those species, and the differences between predator and prey FA signatures due to predator metabolism, it is not possible to visually assess diet composition in a quantitative manner, nor usually even in a qualitative manner. QFASA is the first generation of a statistical model designed to quantitatively estimate predator diet using FA signatures (Iverson *et al.* 2004). QFASA is based on the principles that marine fishes and invertebrates tend to have characteristic FA signatures, that these signatures are deposited in the predator in a predictable way and that by comparing the FA signatures of all potential prey to that of predator fat samples using a statistical model, we can estimate what was eaten.

The term "FA signature" was introduced by Iverson (1993) to simply represent the overall array of all FA in predator or prey, as opposed to the more common historical practice at that time of using only biomarkers or a few selected FA to examine trophic relationships. It is important for investigators to understand that "signature" was never meant to imply a fixed pattern. Variability, sometimes substantial, is inherent in FA signatures, in both predator and prey, in contrast to a "fingerprint" which is considered to be invariable. Thus, the term signature may be somewhat misleading; however, for all practical purposes, the FA composition, FA signature and FA profile of a predator or prey, are all synonymous terms. We may think of FA signatures of prey species as "fixed" (*i.e.*, for estimating the diet of a predator) only in so far as we have empirically characterized these in a given ecosystem and understand (and account for) both between species differences and within species variation. In the same light, what may be prey FA signatures in one study can become predator FA signatures in another study, and vice versa. For instance, FA signatures of prey of pinnipeds and cetaceans can be used to examine the ecology and trophic interactions among marine fishes and invertebrates (*e.g.*, Budge *et al.* 2002, Iverson *et al.* 2002).

Likewise, the FA signatures, once characterized, of pinniped predators, may be used as prey signatures in diet estimation of polar bears that feed on them (Iverson *et al.* 2006).

Details of the initial version of the QFASA model are available in Iverson *et al.* (2004), but briefly, the model proceeds by applying weighting factors ("calibration coefficients") to individual predator FA according to the ways in which they are deposited by the predator. It then takes the average FA signature of each prey species (or within- or among-species group) and estimates the mixture of prey FA signatures that comes closest to matching that of a given calibrated predator's FA stores by minimizing the statistical distance (*e.g.*, Kulback-Liebler) between that prey species mixture and the weighted predator FA profile. The final step is to weight this proportional mixture by the proximate fat content (*i.e.*, relative FA contribution) of each prey species to estimate the proportions in the predator's diet.

QFASA has been shown to provide accurate estimates of diets of individual phocid seals, mink and seabirds, and at time scales relevant to understanding ecological processes (Iverson and Springer 2002; Iverson *et al.* 2003, 2004; Cooper 2004). However, as with any scientific method, QFASA estimates of diet are associated with measurement error and there remain limits to what QFASA can be expected to deliver. Investigators should recognize both the requirements and the limits, as well as the fact that further work remains to be done to improve estimates of diet and to determine those circumstances where the method may not be effective.

The first requirement for using QFASA is appropriate sampling, storage, and FA analysis of predator tissue. As noted previously, it is imperative that investigators sample a metabolically active fat energy reservoir and not structural tissue (*e.g.*, muscle, skin or structural blubber such as the outer layer in cetaceans), which contains highly conserved PL FA and undergoes little FA turnover. The information described in previous sections should allow investigators to assess both the quality of their sample and what their sample represents in relation to the predator's life history or reproductive strategy (*e.g.*, milk *vs.* blubber in capital *vs.* income breeders) and perhaps the time frame of diet information (*e.g.*, blood—last meal, inner blubber—most recent few weeks). Accurate quantification of all FA, and precursors, is also essential.

The second requirement is the appropriate sampling and analysis (both fat content and FA composition) of potential prey species and an understanding of the degree to which prey species can be reliably differentiated by their FA signatures in a given ecosystem. Most importantly, this requires an adequate sample size for each prey species to allow statistical evaluation of within- and between-species variability. While the statistical procedures outlined previously provide useful information about both variability and statistical differences, simulation studies provide a very powerful means with which to test the ability of the QFASA model to estimate diet based solely on differentiating and quantifying prey species by their FA signatures (Iverson *et al.* 2004). These simulations give an indication of the mean reliability of estimates and the noise around those estimates. One can readily see where misidentifications are made (*e.g.*, some prey species that feed very similarly may be frequently mistaken for one another, such as yellowtail flounder and winter flounder) and, based on these, the investigator can decide to group these species together either before or after modeling (*e.g.*, as "flatfish"). Alternatively, there may be significant within-species variability that can be predicted by a factor such as size class (*i.e.*, change in prey diet with age; *e.g.*, Iverson *et al.* 1997, 2002). In this case the investigator can test the separation of prey species "X" into "X small" and "X large" or some other subdivision. We

recommend performing such simulation studies for each prey database prior to any modeling of the diets of predators. Finally, if an important prey species is not present in the database, it obviously cannot be detected in the diet. Thus, while sampling every species in the ecosystem is neither practical nor warranted (*i.e.*, QFASA is unlikely to detect the rare consumption of a prey item), the onus is on the investigator to reasonably sample the range of species that is potentially important to the predator.

The third requirement for using QFASA is an understanding of, and accounting for, the lipid metabolism and deposition in the predator. The relevant issues in predator FA metabolism that need to be considered have all been outlined previously. The critical issue in this regard, assuming all other sampling and analysis criteria are met, is accounting for this metabolism in a quantitative way. Iverson *et al*. (2004) have proposed the use of "calibration coefficients," which are simple weighting factors calculated from long-term diet studies. If an individual predator has been on a diet of a constant FA composition for a long enough period that no effects of previous dietary FA remain, its adipose tissue FA stores would maximally resemble this diet composition, and differences would be due to metabolic processing of individual FA. Thus, a simple division of the amount of a given FA in the predator by the amount in the long-term diet would provide a consistent weighting factor to use for that FA in other diet studies for that type of predator. While these coefficients have proved to be appropriate, similar across species and critical to accurate estimates of diets (Iverson *et al*. 2004), there may be improved ways of accounting for predator metabolism with future work. Further work is also required to assess the affects on these coefficients of factors such as dietary fat content and possible predator-specific differences. For instance, if fat content of prey did affect coefficients, a "reverse" calibration process (*i.e.*, calibrating prey FA signatures rather than predator, based on prey fat content) may be possible. Regardless, these types of calibration studies, with careful attention to controlling dietary FA intake for very long terms, will be important to conduct on different predator types wherever possible.

In the context of the fourth requirement, the need for a quantitative model, the issues of limitations can be discussed. Clearly, a mathematical model is necessary for diet estimation, but there also needs to be appropriate measures of its performance and opportunity for optimization. At present, only one such model exists; there may be future ones developed. Regardless, any such model should include an error component, a measure of the reliability of estimates, and a means by which to capture prey species variability in both fat content and FA composition. As both the FA data and the diet estimates are compositional, and often contain zeros, special techniques are required to handle these situations. Currently, standard errors on the proportions of prey in the estimated diet of an individual predator are being computed using a sequential bootstrap by resampling the prey library to incorporate variability in both the FA signature and fat content of individual prey. Standard errors for a population are derived from the averages of within- and between-individual variation in diet. Other approaches to the calculation of confidence limits are discussed in Stewart (2005). The next issue is goodness of fit (*i.e.*, how does one judge the goodness of the fit of the predator profile and the resulting estimates of diet?). Stewart (2005) has developed a useful method that is analogous to the standard measure used in regression. Nevertheless, the issue of calculating confidence intervals for the estimated diet requires further work. QFASA will always yield an estimated diet and it is important to be aware of the danger of including false positives. False positives will largely be due to prey that have similar FA signatures and the onus is on the

investigator to assess this and interpret results accordingly. Evaluating limits of detection, standard errors around estimates and developing confidence limits on those estimates with QFASA is an important area of research.

Other issues in QFASA include the FA set used in the model and the detection limit of items in the diet. Currently, FA subsets used are based on known biosynthetic and metabolic properties of FA, in addition to their abundance in diets and reliability in analyses (Iverson *et al.* 2004). However, the use of different FA sets can significantly affect diet estimates and needs further optimization. Additionally, it is important to recognize that optimal FA subsets may be species or ecosystem specific; for example, the most useful and abundant dietary FA and isomers can differ between the North Pacific, North Atlantic, and tropical Pacific (Iverson *et al.* 1997, S. J. Iverson[1]) as well as with fresh-water or terrestrial ecosystems (*e.g.*, Smith *et al.* 1996, Iverson *et al.* 2001*b*). Investigators should also recognize that the number of FA contained in the subset used may dictate the number of prey species (or prey groupings) that can be used in modeling, because the number of prey species or groups should not exceed the number of variables (FA) used in modeling.

Finally, the detection limit of prey will depend to some extent on the predator tissue analyzed and, thus, the time frame of the diet inference. Chylomicrons provide information at the scale of hours and the last meal consumed. At this scale, only prey items eaten in that recently consumed single meal would be expected to be estimated in the diet. However, the same quantity of prey consumed in a single meal would not likely be resolved from a sample of predator adipose tissue, which represents a much longer-term integration of diet (*i.e.*, months). Thus, it is important to recognize that while QFASA is able to provide information on those prey that comprise the overall diet of the predator, and those which the predator likely depends upon for survival, it may not be able to detect less common events of prey consumption.

In summary, QFASA is a potentially powerful tool in ecological research that has provided new insight into the foraging patterns and ecology of free-ranging predators, which would otherwise not be possible (*e.g.*, Bowen *et al.* 2006, Iverson *et al.* 2006). Nevertheless, it must be used with knowledge and forethought and the understanding that there are many issues that remain to be resolved.

Specific Issues in the Sampling and Chemical Analysis of FA in the Major Taxa

To put the above discussion into a practical context, we thought it useful to provide some examples of typical analyses and the potential difficulties associated with each (Table 2).

Phocids and Otariids

The analysis of phocid and otariid blubber is perhaps the most straightforward of all marine mammals. Pinniped lipid consists almost entirely of TAG, with no WE or unusual short-chain FA. Some stratification does occur in phocid and otariid blubber, but most species can be captured and temporarily restrained so that an entire blubber core can be obtained. However, recent necropsies of adult male Stellar sea lions

[1] Personal communication from S. J. Iverson, Department of Biology, Dalhousie University, Halifax, Nova Scotia B3H 4J1, Canada, August 2005.

Table 2. Characteristics of selected sample types and complications to consider in sampling and chemical analysis

Sample type	Fat depot sampled	Lipid composition	Site-specific FA composition?	Stratification?
Phocids	Blubber	TAG	No	Limited
Otariids	Blubber	TAG	No	Limited
Cetaceans	Blubber	TAG, WE and short-chain FA	Yes	Yes
Polar bears and sea otters	Subcutaneous adipose	TAG	No	No
Sea birds	Synsacral adipose	TAG[a]	No	No
Planktivorous fish and crustaceans	Entire individual	TAG and WE	NA	NA
Bivalves	Entire individual	TAG and plasmalogens	NA	NA

[a]The adipose tissue of seabirds contains only TAG. However, wax esters are found in the stomach oils and in the uropygial gland (Bishop *et al*. 1983, Roby *et al*. 1986).

(*Eumetopias jubatus*) have revealed an unusual adipose depot below the subcutaneous muscle layer (panniculus) that typically serves as the inner boundary of blubber (D. Tollit[2]). The function of this depot is not yet known, but an obvious role is that of storage of excess fat when individuals are in good body condition. As such, one might expect the FA of this tissue to better mirror that of recent diet; preliminary data suggests that its FA composition is quite similar to that of the inner blubber. Non-blubber adipose layers can also be found below the blubber/muscle interface in the cervical and thoracic regions of many species of cetaceans; preliminary analyses of harbor porpoise samples indicate that, like the sea lions, the composition of this fat layer closely resembles that of the innermost blubber layer (H. N. Koopman, unpublished data). Certainly, further study is necessary to characterize this unusual depot, in terms of both FA composition in relation to diet and its occurrence in age classes and sexes of otariids and other marine mammals.

Cetaceans

Cetaceans may present a number of logistical and analytical problems for FA analysis. Because these animals are entirely pelagic, samples of adipose tissue must be collected either in the form of biopsies taken at sea, or from stranded or by-caught specimens. Because the blubber of many cetacean species exhibits considerable stratification in lipid composition (Fig. 3; Ackman *et al*. 1965, Koopman 2001, Koopman *et al*. 2003), an entire blubber core must be obtained. This can be challenging if remote biopsy techniques are used to collect samples. Unless there is evidence of muscle tissue in the deep portion of the biopsy, there is no certainty as to whether the entire blubber depth has been collected. In addition, there is evidence from some species (*e.g.*, harbor porpoises) that blubber FA stratification increases with the age of

[2] Personal communication from D. Tollit, Marine Mammal Research Unit, Fisheries Centre, University of British Columbia, Vancouver, British Columbia V6T 1Z4, September 2004.

an individual (Koopman *et al*. 1996) and can also be influenced by reproductive status and body condition (Koopman 2001, Koopman *et al*. 2003). Such variation suggests that it is imperative to know that inner blubber has been sampled. For most species, this has not yet been possible for biopsies that are collected at sea with remote techniques, and this must be considered in the interpretation of data. Full-depth samples can readily be collected from dead animals, but it is very important that freshly dead specimens are used and that issues such as postmortem degradation of tissues, health status and body condition of specimens are considered. We consider samples collected more than 1–2 d postmortem from animals stored at non-freezing temperatures to be questionable. In warm climates, or if carcasses are exposed to prolonged direct sunlight, samples should be collected <12 h postmortem to ensure tissue quality and integrity.

Assuming representative and fresh blubber samples can be collected, the chemical analysis of some cetacean fat is potentially more difficult than that of pinnipeds. For example, the adipose tissues of the toothed whales (odontocetes) often contain very short-chain FA, such as *i*-4:0 and *i*-5:0, and/or WE. Methods for dealing with those lipids have been outlined previously; however, additional challenges arise when both short-chain FA and WE are present in the same sample. The methods presented here for oxidizing the alcohols generated from the WE will result in overwhelming loss of short-chain FA. Although time-consuming, techniques such as low temperature TLC and the use of different solvents and minimal volumes can prevent those losses, but are beyond the scope of this review.

Polar Bears, Sea Otters, and Seabirds

Polar bears, sea otters, and seabirds all store body fat as TAG in non-modified adipose tissue rather than blubber, making issues of lipid structure and stratification less important. It is nevertheless essential to sample the adipose tissue sites that represent the non-specialized energy storage reservoirs. Although work has not yet been done with sea otters, we expect them to be similar to polar bears, mink, and seabirds, for which samples collected from several subcutaneous or mysenteric adipose tissue sites within an individual have been found to be identical (Layton *et al*. 2000, Iverson and Springer 2002, Thiemann *et al*. 2006). Thus, for polar bears and sea otters, any large subcutaneous reservoir can be sampled, without concerns about the presence of WE or short-chain FA.

In seabirds, the synsacral fat is generally most easily sampled, but sampling site will depend upon the individual species and the location of their fat reserves. Despite often having a diet high in WE, the adipose tissue of seabirds contains only TAG. However, WE are found in the stomach and its oils, and in the uropygial gland (Bishop *et al*. 1983, Roby *et al*. 1986). Any investigation of the lipid composition at those sites will require an oxidation step to form FA from the fatty alcohols. The major difficulty in seabird sampling is the collection of a sample of sufficient size for reliable analysis. With small or lean birds, it may be especially difficult to collect sufficient samples of fat (*e.g.*, Dahl *et al*. 2003). A minimum sample mass for reliable results is approximately 10 mg, but analysis of smaller samples is possible if care is exercised in the lab workup. Because seabird adipose tissue contains fewer structural elements than blubber, and does not appear to exhibit any stratification in composition, small samples can be assumed to be representative of the entire lipid depot. In this case, direct transesterification may be an appropriate technique.

Planktivorous Fish, Crustaceans, and Bivalves

WE have been found in a variety of fish and crustacean species. Thus, the FAME chromatograms of such prey should be examined carefully for an irregularly rising baseline indicative of alcohols. Bivalves are known to contain plasmalogens and FAME analysis will inevitably produce DMA. Any analysis of bivalves should incorporate an initial TLC separation of the FAME solution to isolate these DMA and save instrument time. In addition, bivalves commonly contain NMI FA. These FA do not present any difficulties in the chemical workup but care must be taken to correctly identify them in the chromatogram.

Summary

FA signature analysis has the potential to be a very powerful technique in both qualitative and quantitative investigations in many areas of ecology, particularly in trophic studies. In this review, we have attempted to provide both general guidelines, as well as detailed procedures, to follow for accurate analysis while avoiding some common pitfalls. The underlying message is, in all cases, to use common sense and prudence in sample collection, preparation, analysis, and interpretation. Some understanding of the protocols described here will allow biologists to make logical decisions regarding the course to follow in collecting and analyzing samples and in interpreting resulting data.

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LITERATURE CITED

AAREFJORD, H., A. J. BJØRGE, C. C. KINZE AND I. LINDSTEDT. 1995. Diet of harbour porpoises (*Phocoena phocoena*) in Scandinavian waters. Report of the International Whaling Commission (Special Issue 16):211–222.

ACKMAN, R. G. 1986. WCOT (capillary) gas-liquid chromatography. Pages 137–206 *in* R. J. Hamilton and J. B. Rossel, eds. Analysis of oils and fats. Elsevier, London, UK.

ACKMAN, R. G. 1989. Fatty acids. Pages 103–137 *in* R. G. Ackman, ed. Marine biogenic lipids, fats, and oils. Volume 1. CRC Press, Boca Raton, FL.

ACKMAN, R. G. 1994. Animal and marine lipids. Pages 292–328 *in* B. S. Kamel and Y. Kakuda, eds. Technological advances in improved and alternative sources of lipids. Chapman and Hall, London, UK.

ACKMAN, R. G. 1998. Comparison of lipids in marine and freshwater organisms. Pages 263–298 *in* M. T. Arts and B. C. Wainman, eds. Lipids in freshwater ecosystems. Springer-Verlag, New York, NY.

ACKMAN, R. G., AND C. A. EATON. 1966. Lipids of the fin whale (*Balaenoptera physalus*) from north Atlantic waters. III. Occurrence of eicosenoic and docosenoic fatty acids in the zooplankter *Meganyctiphanes norvegica* (M. Sars) and their effect on whale oil composition. Canadian Journal of Biochemistry 44:1561–1566.

ACKMAN, R. G., C. A. EATON AND P. M. JANGAARD. 1965. Lipids of the fin whale (*Balaenoptera physalus*) from North Atlantic waters I. Fatty acid composition of whole blubber and blubber sections. Canadian Journal of Biochemistry 43:1513–1520.

ACKMAN, R. G., S. N. HOOPER, S. EPSTEIN AND M. KELLEHER. 1972. Wax esters of barracudina lipid: A potential replacement for sperm whale oil. Journal of the American Oil Chemists' Society 49:378–382.

AITCHISON, J. 1986. The statistical analysis of compositional data. Chapman and Hall, New York, NY.

BACHORIK, P. S. 1982. Collection of blood samples for lipoprotein analysis. Clinical Chemistry 28:1375–1378.

BANDI, Z. L., AND H. K. MANGOLD. 1973. Substrate specificity of enzymes catalyzing interconversions of long-chain acids and alcohols in the rat. FEBS Letters 31:97–100.

BECK, C. A., W. D. BOWEN AND S. J. IVERSON. 2003. Sex differences in the seasonal patterns of energy storage and expenditure in a phocid seal. Journal of Animal Ecology 72:280–291.

BECK, C. A., S. J. IVERSON AND W. D. BOWEN. 2005. Blubber fatty acids of gray seals reveal sex differences in the diet of a size-dimorphic marine carnivore. Canadian Journal of Zoology 83:377–388.

BEST, N. J., C. J. BRADSHAW, M. A. HINDELL AND P. D. NICHOLS. 2003. Vertical stratification if fatty acids in the blubber of southern elephant seals (*Mirounga leonine*): Implications for diet analysis. Comparative Biochemistry Physiology B 134:253–263.

BISHOP, D. G., D. A. RITZ, G. W. HOSIE, J. R. KENRICK AND J. OLLEY. 1983. Fatty acid composition of the lipids of *Puffinus Tenuirostris* (Temminck) in relation to its diet. Journal of Experimental Marine Biology and Ecology 71:17–26.

BLIGH, E. G., AND W. J. DYER. 1959. A rapid method of total lipid extraction and purification. Canadian Journal of Biochemistry and Physiology 37:911–917.

BÖER, M., C. GANNEFORS, G. KATTNER, M. GRAEVE, H. HOP AND S. FALK-PETERSEN. 2005. The arctic pteropod *Clione limacine*: Seasonal lipid dynamics and life-strategy. Marine Biology 147:707–717.

BOWDEN, K., I. M. HEILBRON, E. R. H. JONES AND B. C. WEEDON. 1946. Researches on acetylenic compounds. Part I. The preparation of acetylenic ketones by oxidation of acetylenic carbinols and glycols. Journal of the Chemical Society 39–45.

BOWEN, W. D., S. J. IVERSON, D. J. BONESS AND O. T. OFTEDAL. 2001. Foraging effort, food intake, and lactation performance depend on maternal mass in a small phocid seal. Functional Ecology 15:325–334.

BOWEN, W. D., C. A. BECK, S. J. IVERSON, D. AUSTIN AND J. I. MCMILLAN. 2006. Linking predator foraging behaviour and diet with variability in continental shelf ecosystems: Grey seals of eastern Canada. Pages 63–81 *in* I. L. Boyd, S. Wanless and C. J. Camphuysen, eds. Top predators in marine Ecosystems: Their role in monitoring and Management. Cambridge University Press, Cambridge, U.K.

BRADSTREET, M. S. W. 1980. Thick-billed murres and black guillemots in the Barrow Strait area, N. W. T., during spring: Diets and food availability along ice edges. Canadian Journal of Zoology 58:2120–2140.

BRINDLEY, D. N. 1978. Regulation of fatty acid esterification in tissue. Pages 31–39 *in* R. Dils and J. Knudsen, eds. Regulation of fatty acid and glycerolipid metabolism. Pergamon, Oxford, UK.

BRINDLEY, D. N. 1991. Metabolism of triacylglycerols. Pages 171–203 *in* D. E. Vance and J. Vance, eds. Biochemistry of lipids, lipoproteins and membranes. Elsevier Science, New York, NY.

BROWN, D. J., I. L. BOYD, G. C. CRIPPS AND P. J. BUTLER. 1999. Fatty acid signature analysis from the milk of Antarctic fur seals and southern elephant seals from South Georgia: Implications for diet determination. Marine Ecology Progress Series 187:251–263.

BUDGE, S. M., AND S. J. IVERSON. 2003. Quantitative analysis of fatty acid precursors in marine samples: Direct conversion of wax ester alcohols and dimethylacetals to fatty acid methyl esters. Journal of Lipid Research 44:1802–1807.

BUDGE, S. M., C. C. PARRISH AND C. H. MCKENZIE. 2001. Fatty acid composition of phytoplankton, settling particulate matter and sediments at a sheltered bivalve aquaculture site. Marine Chemistry 76:285–303.

BUDGE, S. M., S. J. IVERSON, W. D. BOWEN AND R. G. ACKMAN. 2002. Among- and within-species variation in fatty acid signatures of marine fish and invertebrates on the Scotian Shelf, Georges Bank and southern Gulf of St. Lawrence. Canadian Journal of Fisheries and Aquatic Sciences 59:886–898.

BUDGE, S. M., M. H. COOPER AND S. J. IVERSON. 2004. Demonstration of the deposition and modification of dietary fatty acids in pinniped blubber using radiolabeled precursors. Physiological and Biochemical Zoology 77:682–687.

CABRINI, L., L. LANDI, C. STEFANELLI, V. BARZANTI AND A. M. SECHI. 1992. Extraction of lipids and lipophilic antioxidants from fish tissues: A comparison among different methods. Comparative Biochemistry and Physiology B 101:383–386.

CANTELLOPS, D., A. P. REID, R. R. EITENMILLER AND A. R. LONG. 1999. Determination of lipids in infant formula powder by direct extraction methylation of lipids and fatty acid methyl esters (FAME) analysis by gas chromatography. Journal of the AOAC International 82:1128–1139.

CARROLL, G. M., J. C. GEORGE, L. F. LOWRY AND K. O. COYLE. 1987. Bowhead whale (*Balaena mysticetus*) feeding near Point Barrow, Alaska, during the 1985 migration. Arctic 40:105–110.

CHAPELLE, S., AND A. A. BENSON. 1986. Studies on the plasmalogens of crustacean gills. Comparative Biochemistry and Physiology B 85:507–510.

CHAPMAN, M. J., AND J. P. KANE. 1975. Stability of the apoprotein of human serum low density lipoprotein: Absence of endogenous endopeptidase activity. Biochemical and Biophysical Research Communications 66:1030–1036.

CHRISTIE, W. W. 1982. Lipid Analysis. Pergamon Press, Oxford, UK.

CHRISTIE, W. W. 1989. Gas chromatography and lipids. A practical guide. Oily Press, Ayr, Scotland.

CHRISTIE, W. W. 1998. Gas chromatography-mass spectrometry methods for structural analysis of fatty acids. Lipids 33:343–353.

CHRISTIE, W. W., E. Y. BRECHANY, S. B. JOHNSON AND R. T. HOLMAN. 1986. A comparison of pyrrolidide and picolinyl ester derivatives for the identification of fatty acids in natural samples by gas chromatography-mass spectrometry. Lipids 21:657–661.

COOK, H. W. 1991. Fatty acid desaturation and chain elongation in eucaryotes. Pages 141–169 *in* D. E. Vance and J. Vance, eds. Biochemistry of lipids, lipoproteins and membranes. Elsevier Science, New York, NY.

COOPER, M. H. 2004. Fatty acid metabolism in marine carnivores: Implications for quantitative estimation of predator diets. Ph.D. thesis, Dalhousie University, Halifax, Canada. 228 pp.

COOPER, M. H., S. J. IVERSON AND H. HERAS. 2005. Dynamics of blood chylomicron fatty acids in a marine carnivore: Implications for lipid metabolism and quantitative estimation of predator diets. Journal of Comparative Physiology B 175:133–145.

COOPER, M. H., S. J. IVERSON AND K. ROUVINEN-WATT. 2006. Metabolism of dietary cetoleic acid (22:1n-11) in mink (*Mustela vison*) and grey seals (*Halichoerus grypus*) studied using radiolabelled fatty acids. Physiological and Biochemical Zoology 79:820–826.

DAHL, T. M., C. LYDERSEN, K. M. KOVACS, S. FALK-PETERSEN, J. SARGENT, I. GJERTZ AND B. GULLIKSEN. 2000. Fatty acid composition of the blubber in white whales (*Delphinapterus leucas*). Polar Biology 23:401–409.

DAHL, T. M., S. FALK-PETERSEN, J. SARGENT, G. GABRIELSEN, H. HOP AND R. M. MILLAR. 2003. Lipids and stable isotopes in common eider, black-legged kittiwake and northern fulmar—a trophic study from an Arctic fjord. Marine Ecology Progress Series 256:257–269.

DALSGAARD, J., M. ST JOHN, G. KATTNER, D. MÜLLER-NAVARRA AND W. HAGEN. 2003. Fatty acid trophic markers in the pelagic marine environment. Advances in Marine Biology 46:225–340.

DOIDGE, D. W. 1990. Integumentary heat loss and blubber distribution in the beluga, *Delphinapterus leucas*, with comparisons to the narwhal, *Monodon monoceros*. Pages 129–140 *in* T. G. Smith, D. J. St. Aubin and J. R. Geraci, eds. Advances in research on the beluga whale, *Delphinapterus leucas*. Canadian Bulletin of Fisheries and Aquatic Sciences, 224.

FALK-PETERSEN, S., T. HAUG, K. T. NILSSEN, A. WOLD AND T. M. DAHL. 2004. Lipids and trophic linkages in harp seal (*Phoca groenlandica*) from the eastern Barents Sea. Polar Research 23:43–50.

FOLCH, J., M. LEES AND G. H. SLOANE-STANLEY. 1957. A simple method for the isolation and purification of total lipids from animal tissues. Journal of Biological Chemistry 226:497–509.

GERACI, J. R., AND V. J. LOUNSBURY. 1993. Marine mammals ashore: A field guide for strandings. Texas A&M Sea Grant Publication, Galveston, TX.

GOEDKOOP, W., L. SONESTEN, G. AHLGREN AND M. BOBERG. 2000. Fatty acids in profundal benthis invertebrates and their major food resources in Lake Erken, Sweden: Seasonal variation and trophic indications. Canadian Journal of Fisheries and Aquatic Sciences 57:2267–2279.

GRAHL-NIELSEN, O., AND O. MJAAVATTEN. 1991. Dietary influence on fatty acid composition of blubber fat of seals as determined by biopsy: A multivariate approach. Marine Biology 110:59–64.

GROB, R. L. 1995. Modern practices of gas chromatography. Wiley and Sons, New York, NY.

GROSCOLAS, R. 1990. Metabolic adaptations to fasting in emperor and king penguins. Pages 269–295 *in* L. S. Davis and J. T. Darby, eds. Penguin Biology. Acedemic Press, New York, NY.

GUILLOU, A., P. SOUCY AND M. KHALIL. 1996. Preparation of fatty acid methyl esters from brook charr tissues: Comparison of a classical and a direct method. Fett Lipid 98:18–21.

GUNSTONE, F. D., J. L. HARWOOD AND F. B. PADLEY. 1986. The lipid handbook. Chapman and Hall, London, UK.

GURR, M. I., AND J. L. HARWOOD. 1991. Lipid biochemistry: An introduction. Chapman and Hall, New York, NY.

HARWOOD, J. L., AND N. J. RUSSELL. 1984. Lipids in plants and microbes. Allen and Unwin, London, UK.

HERMAN, D. P., D. G. BURROWS, P. R. WADE, J. W. DURBAN, C. O. MATKIN, R. G. LE DUC, L. G. BARRETT-LENNARD AND M. M. KRAHN. 2005. Feeding ecology of eastern North Pacific killer whales *Orcinus orca* from fatty acid, stable isotope and organochlorine analyses of blubber biopsies. Marine Ecology Progress Series 302:275–291.

HOBSON, K. A. 1993. Trophic relationships among high Arctic seabirds: Insights from tissue-dependent stable-isotope models. Marine Ecology Progress Series 95:7–18.

HOOKER, S. K., S. J. IVERSON, P. OSTROM AND S. C. SMITH. 2001. Diet of northern bottlenose whales inferred from fatty-acid and stable-isotope analyses of biopsy samples. Canadian Journal of Zoology 79:1442–1454.

HORGAN, I. E., and J. A. BARRETT. 1985. The use of lipid profiles in comparing the diet of seabirds. Pages 493–497 *in* W. R. Siegfried, P. R. Condy, and R. M. Laws, eds. Antarctic nutrient cycles and food webs. Springer-Verlag, Berlin, Germany.

HORROCKS, L. A. 1972. Content, composition, and metabolism of mammalian and avian lipids that contain ether groups. Pages 177–272 *in* F. Snyder, ed. Ether lipids: Chemistry and biology. Academic Press, New York, NY.

IVERSON, J. L., and A. J. SHEPPARD. 1977. Butyl ester preparation for gas-liquid chromatographic determination of fatty acids in butter. Journal of the AOAC 60:284–288.

IVERSON, S. J. 1988. Composition, intake and gastric digestion of milk lipids in pinnipeds. Ph.D. thesis, University of Maryland, College Park, MD. 198 pp.

IVERSON, S. J. 1993. Milk secretion in marine mammals in relation to foraging: Can milk fatty acids predict diet? Symposium of the Zoological Society of London 66:263–291.

IVERSON, S. J. 2002. Blubber. Pages 107–112 *in* W. F. Perrin, B. Würsig and H. G. M. Thewissen, eds. Encyclopedia of marine mammals. Academic Press, San Diego, CA.

IVERSON, S. J., AND O. T. OFTEDAL. 1992. Fatty acid composition of black bear (*Ursus americanus*) milk during and after the period of winter dormancy. Lipids 27:940–943.

IVERSON, S. J., AND A. M. SPRINGER. 2002. Estimating seabird diets using fatty acids: Protocol development and testing of ReFER hypotheses. Report to the National Pacific Marine Research Program. University of Alaska, Fairbanks, AK.

IVERSON, S. J., J. SAMPUGNA AND O. T. OFTEDAL. 1992. Positional specificity of gastric hydrolysis of n-3 polyunsaturated fatty acids of seal milk triglycerides. Lipids 27:870–878.

IVERSON, S. J., O. T. OFTEDAL, W. D. BOWEN, D. J. BONESS AND J. SAMPUGNA. 1995. Prenatal and postnatal transfer of fatty aicds from mother to pup in the hooded seal. Journal of Comparative Physiology B 165:1–12.

IVERSON, S. J., J. P. Y. ARNOULD AND I. L. BOYD. 1997*a*. Milk fatty acid signatures indicate both major and minor shifts in the diet of lactating Antarctic fur seals. Canadian Journal of Zoology 75:188–197.

IVERSON, S. J., K. J. FROST AND L. F. LOWRY. 1997*b*. Fatty acid signatures reveal fine scale structure of foraging distribution of harbor seals and their prey in Prince William Sound, Alaska. Marine Ecology Progress Series 151:255–271.

IVERSON, S. J., S. L. C. LANG AND M. H. COOPER. 2001*a*. Comparison of the Bligh and Dyer and Folch methods for total lipid determination in a broad range of marine tissue. Lipids 36:1283–1287.

IVERSON, S. J., J. E. MCDONALD AND L. K. SMITH. 2001*b*. Changes in the diet of free ranging black bears in years of contrasting food availability revealed through milk fatty acids. Canadian Journal of Zoology 79:2268–2279.

IVERSON, S. J., K. J. FROST AND S. L. C. LANG. 2002. Fat content and fatty acid composition of forage fish and invertebrates in Prince William Sound, Alaska: Factors contributing to among and within species variability. Marine Ecology Progress Series 241:161–181.

IVERSON, S. J., B. S. STEWART AND P. K. YOCHEM. 2003. Captive validation and calibration of fatty acid signatures in blubber as indicators of prey in Hawaiian monk seal diet. NOAA Technical Report, San Diego, CA, 22 pp.

IVERSON, S. J., C. FIELD, W. D. BOWEN AND W. BLANCHARD. 2004. Quantitative fatty acid signature analysis: A new method of estimating predator diet. Ecological Monographs 74:11–235.

IVERSON, S. J., I. STIRLING AND S. L. C. LANG. 2006. Spatial and temporal variation in the diets of polar bears across the Canadian Arctic: Link with and indicators of changes in prey populations. Pages 98–117 *in* I. L. Boyd, S. Wanless and C. J. Camphuysen, eds. Top predators in marine Ecosystems: Their role in monitoring and Management. Cambridge University Press, Cambridge, U.K.

JOSEPH, J. D. 1982. Lipid composition of marine and estuarine invertebrates. Part II: Mollusca. Progress in Lipid Research 21:109–153.

KÄKELÄ, R., AND H. HYVÄRINEN. 1996. Site-specific fatty acid composition in adipose tissues of several northern aquatic and terrestrial mammals. Comparative Biochemistry and Physiology B 115:501–514.

KATTNER, G., C. ALBERS, M. GRAEVE AND S. B. SCHNACK-SCHIEL. 2003. Fatty acid and alcohol composition of the small polar copepods Oithona and Oncaea: Indication of feeding modes. Polar Biology 26:666–671.

KIRSCH, P. E., S. J. IVERSON AND W. D. BOWEN. 2000. Effect of a low-fat diet on body composition and blubber fatty acids of captive juvenile harp seals (*Phoca groenlandica*). Physiological and Biochemical Zoology 73:45–59.

KOOPMAN, H. N. 1998. Topographical distribution of the blubber of harbour porpoises (*Phocoena phocoena*). Journal of Mammalogy 79:260–270.

KOOPMAN, H. N. 2001. The structure and function of the blubber of odontocetes. Ph.D. thesis, Duke University, Durham, NC. 408 pp.

KOOPMAN, H. N., S. J. IVERSON AND D. E. GASKIN. 1996. Stratification and age-related differences in blubber fatty acids of the male harbour porpoise (*Phocoena phocoena*). Journal of Comparative Physiology, B 165:628–639.

KOOPMAN, H. N., S. J. IVERSON AND A. J. READ. 2003. High concentrations of isovaleric acid in the fats of odontocetes: Stability in the melon vs. variation and patterns of accumulation in blubber. Journal of Comparative Physiology B 173:247–261.

KOOPMAN, H. N., S. M. BUDGE, D. R. KETTEN AND S. J. IVERSON. 2006. The topographical distribution of lipids inside the mandibular fat bodies of odontocetes: Remarkable complexity and consistency. Journal of Oceanographic Engineering Special Issue on Effects of Sound in the Marine Environment 31:95–106.

LAYTON, H., K. ROUVINEN AND S. J. IVERSON. 2000. Body composition in mink (*Mustela vison*) kits during 21–42 days postpartum using estimates of hydrogen isotope dilution and direct carcass analysis. Comparative Biochemistry and Physiology A 126:295–303.

LIN, J.-H., L.-Y. LIU, M.-H. YANG AND M.-H. LEE. 2004. Ethyl acetate/ethyl alcohol mixtures as an alternative to Folch reagent for extracting animal lipids. Journal of Agricultural and Food Chemistry 52:4984–4986.

LITCHFIELD, C., A. J. GREENBERG AND J. G. MEAD. 1976. The distinctive character of Ziphiidae head and blubber fats. Cetology 23:1–10.

LITCHFIELD, C., A. J. GREENBERG, R. G. ACKMAN AND C. A. EATON. 1978. Distinctive medium chain wax esters, triglycerides, and diacyl glyceryl ethers in the head fats of the Pacific Beaked Whale, *Berardius bairdi*. Lipids 13:860–866.

LIU, K.-S. 1994. Preparation of fatty acid methyl esters for gas-chromatographic analysis of lipids in biological materials. Journal of the American Oil Chemists' Society 71:1179–1187.

LOWRY, L. F. 1993. Foods and feeding ecology. Pages 201–238 *in* J. J. Burns, J. J. Montague, and C. J. Cowles, eds. The bowhead whale, Special Publication No. 2, Society for Marine Mammalogy. Allen Press, Lawrence, KS.

MAYZAUD, P., E. ALBESSARD, P. VIRTUE AND M. BOUTOUTE. 2000. Environmental constraints on the lipid composition and metabolism of euphausiids: The case of *Euphausia superba* and *Meganyctiphanes norvegica*. Canadian Journal of Fisheries and Aquatic Sciences 57:91–103.

MORII, H., AND T. KANEDA. 1982. Biosynthesis of branched-chain fatty acids from branched-chain amino acids in subcutaneous tissue of the marine little toothed whale, *Stenella caeruleo-alba*. Comparative Biochemistry and Physiology B 71:357–365.

NAJDEK, M., S. PUKARIC AND A. B. BOCHDANSKY. 1994. Contribution of zooplankton lipids to the flux of organic matter in the northern Adriatic Sea. Marine Ecology Progress Series 111:241–249.

NERINI, M. 1984. A review of gray whale feeding ecology. Pages 423–450 *in* M. L. Jones, S. L. Swartz and S. Leatherwood, eds. The grey whale, *Eschrichtius robustus*. Academic Press, Orlando, FL.

NORSETH, J. AND B. O. CHRISTOPHERSEN. 1978. Chain shortening of erucic acid in isolated liver cells. FEBS Letters 88:353–357.

OFTEDAL, O. T. 1997. Lactation in whales and dolphins: Evidence of divergence between baleen- and toothed-species. Journal of Mammary Gland Biology and Neoplasia 2:205–230.

OSMUNDSEN, H., C. E. NEAT AND K. R. NORUM. 1979. Peroxisomal oxidation of long chain fatty acids. FEBS Letters 99:292–296

PABST, D. A. 1996. Springs in swimming animals. American Zoologist 36:723–735.

PALTAUF, F. 1994. Ether lipids in biomembranes. Chemistry and Physics of Lipids 74:101–139.

PAPAMANDJARIS, A. A., D. E. MACDOUGALL AND P. J. H. JONES. 1998. Medium chain fatty acid metabolism and energy expenditure: Obesity treatment implications. Life Sciences 62:1203–1215.

PASCAL, J. C., AND R. G. ACKMAN. 1975. Occurrence of 7-methyl-7-hexadecenoic acid, the corresponding alcohol, 7-methyl-6-hexadecenoic acid, and 5-methyl-4-tetradecenoic acid in sperm whale oils. Lipids 10:478–482.

PHLEGER, C. F., P. D. NICHOLS AND P. VIRTUE. 1997. The lipid, fatty acid and fatty alcohol composition of the myctophid fish *Electrona antarctica*: High levels of wax esters and food chain implications. Antarctic Science 9:258–265.

PHLEGER, C. F., M. M. NELSON, B. D. MOONEY AND P. D. NICHOLS. 1999. Wax esters versus triacylglycerols in myctophid fishes from the Southern Ocean. Antarctic Science 11:436–444.

POND, C. M. 1998. The fats of life. Cambridge University Press, Cambridge, UK.

POND, C. M., C. A. MATTACKS, R. H. COLBY AND M. A. RAMSAY. 1992. The anatomy, chemical composition, and metabolism of adipose tissue in wild polar bears (*Ursus maritimus*). Canadian Journal of Zoology 70:326–341.

POND, C. M., C. A. MATTACKS AND P. PRESTRUD. 1995. Variability in the distribution and composition of adipose tissue in arctic foxes (*Alopex lagopus*) on Svalbard. Journal of Zoology, London 236:593–610.

RACLOT, T. 2003. Selective mobilization of fatty acids from adipose tissue triacylglycerols. Progress in Lipid Reseach 42:257–288.

RACLOT, T., R. GROSCOLAS AND Y. CHEREL. 1998. Fatty acid evidence for the importance of myctophid fishes in the diet of king penguins, *Aptenodytes patagonicus*. Marine Biology 132:523–533.

RATNAYAKE, W. N., AND R. G. ACKMAN. 1979. Fatty alcohols in capelin, herring and mackerel oils and muscle lipids: 1. Fatty alcohol details linking dietary copepod fat with certain fish depot fats. Lipids 14:795–803.

RECCHIA, C. A., AND A. J. READ. 1989. Stomach contents of harbour porpoises, *Phocoena phocoena* (L.), from the Bay of Fundy. Canadian Journal of Zoology 67:2140–2146.

REEVES, R. R., B. S. STEWART, P. J. CLAPHAM AND J. A. POWELL. 2002. National Audobon Society Guide to Marine Mammals of the World. Alfred A. Knopf, New York, NY.

ROBY, D. D., A. R. PLACE AND R. E. RICKLEFS. 1986. Assimilation and deposition of wax esters in planktivorous seabirds. Journal of Experimental Zoology 238:29–41.

ROOD, D. 1995. A practical guide to the care, maintenance, and troubleshooting of capillary gas chromatographic systems. Hüthig, Heidelberg, Germany.

ROUVINEN, K., AND T. KIISKINEN. 1989. Influence of dietary fat source on the body fat composition of mink (*Mustela vison*) and blue fox (*Alopex lagopus*). Acta Agricultura Scandinavia 39:279–288.

RYG, M., T. G. SMITH AND N. A. ØRITSLAND. 1988. Thermal significance of the topographical distribution of blubber in ringed seals (*Phoca hispida*). Canadian Journal of Fisheries and Aquatic Sciences 45:985–992.

SANTOS, M. B., AND G. J. PIERCE. 2003. The diet of harbour porpoises (*Phocoena phocoena*) in the Northeast Atlantic. Oceanographic and Marine Biology—an Annual Review 41:355–390.

SARGENT, J. R. 1976. The structure, metabolism and function of lipids in marine organisms. Pages 149–212 *in* D. C. Malins and J. R. Sargent, eds. Biochemical and biophysical perspectives in marine biology. Academic Press, London, UK.

SARGENT, J. R., R. F. LEE AND J. C. NEVENZEL. 1976. Marine waxes. Pages 49–91 *in* P. E. Kolattukudy, ed. Chemistry and biochemistry of natural waxes. Elsevier, Oxford, UK.

SARGENT, J. R., R. J. PARKES, I. MUELLER-HARVEY AND R. J. HENDERSON. 1987. Lipid biomarkers in marine ecology. Pages 119–138 *in* M. A. Sleigh, ed. Microbes in the sea. Wiley and Sons, New York, NY.

SHAIKH, N. A. 1986. Extraction, purification, and analysis of lipids from animal tissues. Pages 289–302 *in* H. A. Fozzard, E. Haber, R. B. Jennings, A. M. Katz and H. E. Morgan, eds. The heart and cardiovascular system. Raven Press, New York, NY.

SMEDES, F. 1999. Determination of total lipid using non-chlorinated solvents. Analyst 124:1711–1718.

SMEDES, F., AND T. K. ASKLAND. 1999. Revisiting the development of the Bligh and Dyer total lipid determination method. Marine Pollution Bulletin 38:193–201.

SMITH, R. J., K. A. HOBSON, H. N. KOOPMAN, AND D. M. LAVIGNE. 1996. Distinguishing between populations of fresh- and salt-water harbour seals (*Phoca vitulina*) using stable isotope ratios and fatty acid profiles. Canadian Journal of Fisheries and Aquatic Sciences 53:272–279.

SMITH, S. J., S. J. IVERSON AND W. D. BOWEN. 1997. Fatty acid signatures and classification trees: New tools for investigating the foraging ecology of seals. Canadian Journal of Fisheries and Aquatic Sciences 54:1377–1386.

STENSTROM, M. K., S. FAM AND G. S. SILVERMAN. 1986. Analytical methods for quantitative and qualitative determination of hydrocarbons and oil and greases in water and wastewater. Environmental Technology Letters 7:625–636.

STEVENS, J. 1986. Applied multivariate statistics for the social sciences. Lawrence Erlbaum Associates, NJ.

STEWART, C. 2005. Inference on the diet of predators using fatty acid signatures. Ph.D. thesis, Dalhousie University, Halifax, NS; 191 pp.

TABACHNICK, B. G., AND L. S. FIDELL. 1989. Using Multivariate Statistics. Harper Collins Publishers, New York, NY.

THIEMANN, G. W., S. M. BUDGE AND S. J. IVERSON. 2004. Fatty acid composition of blubber: A comparison of *in situ* direct and traditional extraction methods. Marine Mammal Science 20:284–295.

THIEMANN, G. W., S. J. IVERSON AND I. STIRLING. 2006. Seasonal, sexual, and anatomical variability in the adipose tissue composition of polar bears (*Ursus maritimus*). Journal of Zoology London 269:65–76.

UNDELAND, I., M. HÄRRÖD AND H. LINGNERT. 1998. Comparison between methods using low-toxicity solvents for the extraction of lipids from herring (*Clupea harengus*). Food Chemistry 61:355–365.

VARANASI, U., AND D. C. MALINS. 1971. Unique lipids of the porpoise (*Tursiops gilli*): Differences in triacylglycerols and wax esters of acoustic (mandibular canal and melon) and blubber tissues. Biochimica et Biophysica Acta 231:415–418.

VOLPE, J. J., AND P. R. VAGELOS. 1973. Saturated fatty acid biosynthesis and its regulation. Annual Review of Biochemistry 42:21–60.

VOSS, A., M. REINHART, S. SANKARAPPA AND H. SPRECHER. 1991. The metabolism of 7,10,13,16,19-docosapentaenoic acid to 4,7,10,13,16,19-docosahexaenoic acid in rat liver is independent of a 4-desaturase. Journal of Biological Chemistry 266:19995–20000.

WALTON, M. J., R. J. HENDERSON AND P. P. POMEROY. 2000. Use of blubber fatty acid profiles to distinguish dietary differences between grey seals *Halichoerus grypus* from two UK breeding colonies. Marine Ecology Progress Series 193:210–208.

WAMBERG, S., C. R. OLESEN AND H. O. HANSEN. 1992. Influenece of dietary sources of fat on lipid synthesis in mink (*Mustela vison*) mammary tissue. Comparative Biochemistry and Physiology A 103:199–204.

WETZEL, D. L., AND J. E. REYNOLDS. 2004. Definitive identification of fatty acid constituents in marine mammal tissues. Canadian Journal of Fisheries and Aquatic Science 61:554–560.

WEST, G. C., J. J. BURNS AND M. MODAFFERI. 1979. Fatty acid composition of Pacific walrus skin and blubber fats. Canadian Journal of Zoology 57:1249–1255.

ZAR, J. H. 1999. Biostatistical analysis. Prentice-Hall, Upper Saddle River, NJ.

ZWEIG, G., AND J. SHERMA. 1972. CRC handbook of chromatography. CRC Press, Cleveland, OH.

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SUPPLEMENTARY MATERIAL

The following supplementary material is available for this article online:
Supplementary Appendix 1
Supplementary Appendix 2
Supplementary Appendix 3
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