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Diet

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9.1 Introduction

Marine mammals are major consumers within marine food webs and probably have a key role in determining food web structure (Bowen 1997). Understanding their diets is important for quantifying trophic interactions and for supporting a broad range of ecological research. Diet estimation in marine mammals has relied on indirect observation because there are limited opportunities to observe directly what marine mammals eat. Indirect methods of observation estimate the diet from samples using a variety of analytical methods that are subject to bias and measurement error. Thus, the quantitative estimation of diet uses statistical inference and is not merely a description of what kinds of prey are eaten.

Historically, diet has been studied by identifying prey remains recovered from stomachs, intestines, and faeces (also termed scats). This can produce a bewildering array of material from almost every type of marine organism above the size of micro-zooplankton, and these are often represented as prey fragments of varying size and stage of digestion. Methods developed more recently include the comparison of stable isotope (SI) and fatty acid (FA) signatures in the tissues of predator and prey (i.e. biochemical-based methods), and molecular identification of prey using DNA. Visual observation and remote sensing (e.g. animal-borne video and sensors) have also been used (Chapter 11).

Each method has its own strengths and weaknesses and there have been considerable efforts to document these, and especially to account for systematic biases (see Table 9.1; Pierce and Boyle 1991; Santos *et al.* 2001; Pierce *et al.* 2004; Stenson and Hammill 2004; Budge *et al.* 2006; Tollit *et al.* 2006). These efforts have included captive feeding studies and computer simulations (e.g. Harvey 1989; Hammond and Rothery 1996; Hobson *et al.* 1996; Tollit *et al.* 1997; Iverson *et al.* 2004; Deagle *et al.* 2005). None of the current methods can be universally recommended, so the use of combinations of complementary methods is advisable

Table 9.1 *Main methods used to describe diet in marine mammals, with key information on each method's requirements and products with associated strengths and limitations. 'Regression' refers to the relationship between hard part measurement and length or mass of individual prey.*

Method	Animal impact[1]	Diet history	Cost	Identification of prey species	Prey size estimate	Mass percentage (needed for consumption models)	Requirements	Other limitations
Faeces, hard remains	None	Few meals[2]	Low (mod. if all HP used)	Yes (reference collection needed)	Yes (length regressions needed)	Yes (mass regressions needed)	• Reference collection • Regressions • Correction factors for loss and size reduction of HP	• Sex/age of individual generally unknown • Differential prey digestion and retention (prey without HP not represented, prey with fragile HP underestimated, large beaks underestimated) • Special identification skills often required
Regurgitates, hard remains	None	Few meals[2]	Low	Yes (reference collection needed)	Yes (length regressions needed)	Yes (mass regressions needed)	• Reference collection • Regressions	• Sex/age of individual generally unknown • Differential prey digestion and retention (overestimation of large HP)
Prey DNA faeces (stomachs)	None (high[3])	Few meals[2]	Mod.	Yes (genetic data on prey needed)	No	Possible, but expensive and little validation	• Prey primers	• Prey DNA not extractable from all samples • Optimization of primer sets necessary for all prey species • Sensitivity levels poorly known
Stomachs, hard remains	High[3]	Few meals[2]	Low (mod. if all HP used)	Yes (reference collection needed)	Yes (length regressions needed)	Yes (mass regressions needed)	• Reference collection • Regressions • Correction factors for loss and size reduction of HP	• Differential prey digestion and retention (prey with fragile HP underestimated, opposite with large robust HP or beaks) • Information from dead animals, so representation of whole population uncertain • Empty stomachs often reduce sample sizes

Lavage (enema), hard remains	Moderate (low)	Few meals[2]	Mod.	Yes (reference collection needed)	Yes (length regressions needed)	Yes (mass regressions needed)	• Reference collection - regressions	• Large HP difficult to recover • Empty stomachs (colons) • Capture and handling issues
Stable isotopes	Moderate (capture or dart projector)	Days to years depending on the tissue	Low	No (only estimation of trophic level)	No	No	• Isotopic signatures from lower trophic level	• Limited in estimation of prey species • Interpretation of comparison between different environments and time-scales difficult without isotopic signatures from lower trophic level
FA signatures	Moderate (capture or dart projector)	Days–months, depending on the tissue	Mod.	Possible with QFASA (prey FA library and accurate calibrations needed)	No	Possible with QFASA (prey FA library and accurate calibrations needed)	• Full prey FA library and appropriate calibrations for QFASA	• Time frame for blubber only approximate • Variability in FA metabolism not fully understood, potentially resulting in imprecise calibrations and diet estimates • Species not in prey FA library are not identified
Animal-borne camera	Moderate (capture)	Days–weeks	High	Possible	Possible	No	• Suitable-sized camera	• High cost and unit recovery required • Limited feeding events captured
Direct observation	No	Immediate	Low–mod.	Possible	Possible	No	• Suitable viewing platform	• Typically limited to prey brought to the surface • Typically limited to near-shore interactions

[1]Excluding impacts of disturbance; [2]number of meals depends largely on level of retention; [3]stomach analysis requires carcasses.

Abbreviations: HP, hard parts; IS, isotopic signatures; FA, fatty acid; and QFASA, quantitative fatty acid signature analysis; mod., moderate.

(e.g. Hooker *et al*. 2001a; Hammill *et al*. 2005; Herman *et al*. 2005; Tucker *et al*. 2008; Tollit *et al*. 2009). Approaches that provide quantified descriptions of diet (e.g. by mass) are most valuable.

Many marine mammals range widely and exhibit seasonal movements. Thus both the sampling design and the method used should be selected to reflect the temporal and geographical variability in diets and the fact that diet is dynamic, responding in non-linear ways to both intrinsic (e.g. age, sex, condition) and extrinsic (e.g. prey abundance, distribution, energy content) factors.

9.2 Collection of gastrointestinal tract contents

9.2.1 Sampling dead animals

Whenever possible, collection of stomach and intestinal tract contents (Bigg and Fawcett 1985; Pierce and Boyle 1991; Croxall 1993; Ridoux 1994; Santos *et al*. 2001) should be accompanied by morphometric data (see Chapter 5); Geraci and Lounsbury 1993). Appropriate care about infections and other potential health risks needs to be taken when handling gastro-intestinal tracts.

Gastrointestinal tracts often are empty or contain little identifiable food remains, so reducing their value. This can be partly overcome by sampling specimens at sea near foraging grounds. Samples from stranded and by-caught animals have been used for diet analysis, but may not be representative (see Table 9.1; Santos *et al*. 2001). Although these days samples rarely come from hunting, they may be obtained in association with harvesting or from animals being killed as part of a management activity.

Stomachs or the complete digestive tracts should be collected. Individual sections can be ligatured and stored frozen (normally at $-20\,^{\circ}C$, but $-70\,^{\circ}C$ is preferred if DNA is to be extracted at a later stage). Food remains are commonly recovered separately from the oesophagus, stomach, and colon, but the remainder of the intestinal tract often yields little additional material. The stomach contents of large cetaceans are normally removed *in situ*.

Ideally, digestive tracts should be examined while the contents are fresh. However, resistant skeletal structures of prey (termed 'hard parts') can be extracted from stomachs even when carcasses are moderately decomposed, although soft tissues of prey will be degraded. Frozen storage in sealed containers will not harm hard structures, but soft tissues (and fatty acids) will continue to degrade, especially at the temperature used in normal domestic freezers. Storage in alcohol or formalin is not recommended since fixed prey remains are harder to separate, fish otoliths (ear bones) may dissolve or become more friable, and such treatment generally prevents biochemical and molecular analyses.

Stomachs and intestinal tracts should be weighed before and after all material has been removed to determine the mass of contents. To collect the prey remains, a complete median longitudinal incision is made from anterior to posterior. The stomach should be

thoroughly rinsed. Longitudinal folds should be individually reflected and rinsed, the lining should be examined for cephalopod beaks (or sharp bone fragments). For the remaining sections of the digestive tract, remnants should be squeezed out, cut longitudinally, inverted, and washed thoroughly. Prey should be separated into major types (e.g. fish, cephalopods, crustaceans). The digestion condition of each organism or type may be scored (Meynier 2004; Pusineri *et al.* 2007) to allow interpretation of differential susceptibility of prey to digestion (see Section 9.5). Samples should be processed (identified, weighed, and measured) as soon after collection as possible, with a representative sample of macerated flesh stored frozen at −70 °C for future DNA analysis. Parasites should be stored in 95% non-denatured ethanol.

9.2.2 Lavage

Stomach-flushing (lavage) has been used in several pinniped species (Boness *et al.* 1994; Harvey and Antonelis 1994). The percentage of samples with food remains can range between 0 and 92% (Rodhouse *et al.* 1992; Boness *et al.* 1994; van den Hoff *et al.* 2003). To perform lavage, animals are restrained and immobilized. A block of wood or stiff rubber (40 cm × 4 cm × 7 cm) with a 3.5 cm hole is placed into the animal's mouth and the lubricated (surgical lubricant), rounded end of a gastric feeding tube (foal size) or clear semi-flexible PVC tube (internal diameter 2.5 cm, wall thickness 0.5 cm) is carefully inserted in the animal's mouth and gently pushed past the oesophagus into the animal's stomach. A canvas strap can be wrapped around the upper canines to ensure safety and to maintain alignment. If the tube is properly in the stomach it should reach the length measured from the mouth to about the end of the sternum; the end of the tube should be placed deeper in the stomach rather than shallower to avoid reflux into the oesophagus. Approximately 2–3 L of seawater can be passed by gravity (Rodhouse *et al.* 1992) and the free end of the tube subsequently lowered below the seal's head and the sample collected in a fine-mesh sieve. An active suction pump can also be used (Boness *et al.* 1994). Multiple lavages may be needed in some species as <50% of recently fed cephalopod beaks were collected in captive tests after two lavages (Harvey and Antonelis 1994). Cephalopod beaks may be difficult to dislodge. As the size of the remains recovered is limited by the diameter of the tube, the results will generally provide a biased view of the diet. Overall, lavaging is subject to unquantified biases and works best for small or heavily digested prey. The use of emetics to provide regurgitated stomach samples is an approach that is not recommended, both because of the animal welfare issues and due to the biases that can result from incomplete recovery of contents.

9.2.3 Rectal enema and faecal loops

Rectal enemas or faecal loops both aim to obtain a sample of faeces from live-captured animals. Staniland *et al.* (2003) found prey remains in 93% of 149 samples from recently returned female Antarctic fur seals, with no significant differences in the mean krill sizes collected by enema compared to scat sampling. Animals are

typically physically restrained during the procedure (Gentry and Holt 1982). A soft polyethylene hose (12 mm diameter) is connected to a plastic bottle, filled with approximately one litre of warm water, and inserted into the animal's colon. The water is then introduced *via* the hose and one-way valve by gently squeezing the bottle. Once the bottle is empty or the resistance becomes too great, the hose is removed and the material is naturally expelled by the animal into a large plastic tray.

9.3 Collection of faeces and regurgitated/discarded prey remains

The collection of prey remains from scats is the most widely used method to estimate pinniped diets, but it does not appear to be useful for sampling all species of otariids (because many hard remains are regurgitated) (N.J. Gales and Cheal 1992). Scats can often be collected easily and in large numbers. Disturbance can be minimal or mainly short term (Kucey and Trites 2006).

Although easy to collect, without additional DNA analysis (Reed *et al.* 1997), the sex, age, and potentially even the species of the source animal will usually be unknown. Scats represent relatively recent feeding (last few days) and thus presumably feeding in relatively near-shore areas. This may not pose a problem for coastal species, such as the harbour seal (*Phoca vitulina*), but will bias diet estimates of more wide-ranging offshore species, such as elephant seals (*Mirounga angustirostris*), where only a small portion of foraging effort might be near haul-out sites. An indication of the effective sampling area near a collection site may be estimated from data on food passage rates and swimming speeds (Prime and Hammond 1987), or by tracking animals (see Chapter 11). Interspecific differences in prey passage times (Fea and Harcourt 1997; Tollit *et al.* 2003) may also affect the probability of some prey being recovered in scats at haul-out sites. Integrating passage time information together with foraging trip durations and foraging location data can provide an assessment of the level of potential collection biases (Smout 2006).

The collection of cetacean faeces is more challenging and requires boat-based focal follows (see Chapter 12). Dogs have been used to detect samples from up to a nautical mile from the source, and could locate three to five times the number of samples per unit effort than were collected by human observers (Rolland *et al.* 2006). Faeces usually produce a cloud plume of material in the water (pink coloured in the case of krill consumers), and material can be picked up using a fine (500–1000 μm) mesh net (N.J. Gales and Jarman 2002) and extendable pole or, in the case of dolphins, snorkellers can collect sinking faeces in plastic vials (Parsons *et al.* 1999). Collection of fish scales and tissue from killer whale (*Orcinus orca*) predation events has also been successful (Ford and Ellis 2006). Deecke *et al.* (2005) used underwater sounds of kills made by transient killer whales as the cue to collect their prey remains by net.

Scats range in consistency from semi-liquid to solid and can be broken into fragments and spread over a wide area by currents or animal movements. Ultimately,

judgement about which fragments belong together must be based on their location, size, consistency, and colour. Ideally, only entire recently deposited faecal samples should be collected. If dried up, older or partial samples are collected, this should be recorded. Regurgitated samples or prey discards may be collected using the same methods as faecal samples, but should be analysed separately. Scat samples are normally stored frozen at −20 °C, as soon as possible.

Sub-sampling scats for predator or prey DNA (or hormones) is ideally undertaken within 48 h of collection to minimize degradation. If only information on the defecator is needed then scraping 2–3 ml from the outer surface of the scat with a sterile spoon should suffice, otherwise scats should first be homogenized, ideally by mixing the sample with distilled water (∼50 ml) in a jar, leaving overnight, and gently shaking the resulting slurry. If only soft-tissue prey remnants are being used for identification, approximately 10 g of scat homogenate/slurry is then removed and gently pressed through a <0.5 mm plastic mesh. Approximately 2–3 ml of soft scat material scraped from the underside of the mesh can then be stored at either −70 °C or refrigerated with 4–5 times the sample volume of 95% non-denaturing ethanol.

9.4 Sampling bias

The number of samples collected will depend on the questions asked, the method used, and the spatial and temporal scales of interest (Hayes and Steidl 1997; Reed and Blaustein 1997). The number of samples required will also vary in relation to the diet breadth and variability (Arim and Naya 2003; Trites and Joy 2005). Sampling design and the methods used should be selected to reflect the demographic, temporal, and geographical variability in diets. The optimal sample size can be determined if some prior information is available on diet breadth and variability (Lance *et al.* 2001), but conducting a pilot study is recommended. Monte Carlo simulation on the results from pilot samples can be used to determine the sample size required to achieve a predetermined level of precision (Lance *et al.* 2001). Assuming constant proportions of species pass into the scats, Trites and Joy (2005) estimated that 59 scats should be collected to be 95% confident of collecting at least 1 scat containing a species with a 5% probability of occurrence. To statistically distinguish between populations, they found that collecting 59 scats would suffice for diets containing 12 or more exponentially distributed (in terms of frequency of occurrence) species of prey, 94 scats for diets containing 6 or more species, and 179 scats for diets with 3 species. Hammond and Rothery (1996) also used re-sampling techniques to estimate the confidence limits of grey seal (*Halichoerus grypus*) diet estimates and the relative magnitude of different sources of error. Their analysis indicated that a minimum of approximately 100 scats should be collected in each area/season combination, and additionally they highlighted systematic errors due to measurement errors in estimating fish weight from partially digested otoliths.

9.5 Laboratory processing of prey hard structures

The contribution of each prey species to the diet is ideally based on the mass and number of individuals consumed. The reconstructed biomass of prey can then be converted to energy consumed using estimates of prey energy density. Identification of prey remains is time-consuming and requires experience, access to good reference material, and, in some cases (e.g. identifying cephalopod beaks; Clarke 1986), specialist training.

Occurrence indices only require structure identification. A major variant in protocol is whether to use otoliths and beaks alone or to include alternative diagnostic skeletal structures (i.e. all hard parts). The latter approach increases the detection rates for many prey species (Olesiuk *et al.* 1990b; and see Section 9.5.2 below) but it requires excellent taxonomic identification skills and extensive reference material. Few field studies have attempted biomass reconstruction using all hard part structures (Laake *et al.* 2002; Sigler *et al.* 2009), largely due to the scarcity of appropriate allometric regressions and correction factors that aim to take account of the loss in size and number of items due to digestion (Harvey 1989). Laake *et al.* (2002) compared the results of occurrence and biomass reconstruction estimators and found ten-fold differences in species consumption estimates between the two indices for the smallest and largest prey. Sigler *et al.* (2009) highlighted two- to threefold differences. Captive, mixed-diet feeding studies show that it is possible to obtain good biomass estimators using all hard part structures (Tollit *et al.* 2007; Philips and Harvey 2009). Together, these studies highlight the weakness of occurrence indices to quantify diet when diet/scat diversity is high (i.e. generalist predators) and when prey sizes consumed vary considerably (see also Section 9.6).

The processing of GI tract contents, scats, and regurgitates is complicated by the fact that the digestive state of prey is highly variable. Prey remains may come from an unknown number of meals over an unknown period, and different types of food are digested at different rates. For GI tracts, the best approach is one that assesses diet based, initially, on the fresh food fraction alone, as well as a comparison with all prey remains whatever their digestive state. Data from both *in vivo* and recent *in vitro* digestion rate studies can be integrated into analyses to account for the states of digestion (see Sections 9.3 and 9.6; Murie 1987).

9.5.1 Prey extraction

Extraction of prey structures from GI contents, regurgitates, and scats follows the same general procedures. The contents of each section of the GI tract should be poured through up to three or four nested sieves with a minimum mesh size of 0.2–0.355 mm, and washed with water and a soft brush to remove as much soft residue as possible.

Thawed scats should be weighed or a volume noted by measuring the settled level of samples that have been suspended in jars with water, after removing or accounting for any substrate in the case of pinnipeds. Soaking samples in a mild 1% detergent solution can reduce odour and clean up hard parts. Strong detergents may erode or dissolve more friable hard parts and aliquots for any future DNA or hormone analysis should be removed first.

The extraction of prey hard part structures from scats can be done manually, using a water bath, sieving, elutriating, or (with the faecal material inside a mesh bag) a washing machine. Material can be suspended (with 1% detergent) in a sorting tray (35 × 45 cm) and examined. Buoyant parts such as crustacean remains and fish scales can be skimmed, while the dense residue can be examined under a binocular microscope and prey remains picked out manually (Reid 1995). Spray-washing scats through stacked sieves of decreasing mesh size (10, 4.75, 1.0, and 0.5–0.25 mm mesh) and using brushes or soft spatulas to break up hardened material can damage fragile structures. Consequently, Murie and Lavigne (1985) suggested using nested sieves in conjunction with flowing water baths, while Bigg and Olesiuk (1990) developed an elutriator, which is a semi-closed system that separates prey remains from soluble waste material using differences in their densities. The elutriator is efficient, but uses large volumes of water and secondary processing in nested sieves is frequently also necessary. A top-loading washing machine, set on a gentle wash, is recommended (Orr *et al.* 2003) for the bulk processing of approx. 25 scats. Loss rates (∼5%) and size reductions of otoliths (∼1%) using this method were found to be similar to nested sieves, but the processing time was reduced by more than half. Individual scats are placed into one or two labelled, tightly closed, 3.8 L × 124 μm mesh bags.

Some fish have extremely small otoliths—e.g. pipefish (Syngnathinae) otoliths may be lost even using a 0.2 mm sieve, and many other small fish, including gobies (Gobiidae) and sand eels (Ammodytidae), may have otoliths less than 1 mm in breadth. Hard structures for species identification (and enumeration) should be cleaned (immersion in 70% alcohol for some hours will effectively sterilize the material), air-dried on absorbent paper, and then transferred to glass storage vials. Cephalopod beaks, statoliths, lenses and pens, crustacean carapaces and telsons, and other invertebrate remains (which may include polychaete jaws) should be stored in 70–95% non-denatured ethanol or isopropyl alcohol to prevent distortion.

9.5.2 Prey identification

Prey should be identified under a binocular microscope to the lowest possible taxon by comparing with reference material. There are a number of good identification guides of fish structures (Newsome 1977; Härkönen 1986; Rosello Izquierdo 1986; Cannon 1987; Hansel *et al.* 1988; Smale *et al.* 1995; Prenda *et al.* 1997; Watt *et al.* 1997; Harvey *et al.* 2000; Leopold *et al.* 2001; Campana 2004; Tuset *et al.* 2008), as well

as extensive archaeozoological (see Casteel 1976) and osteological (e.g. Norden 1961; Mujib 1967; Boschi *et al.* 1992) literature. Less information is available to assist the identification of crustaceans (e.g. Mori *et al.* 1992) or cephalopods (e.g. Clarke 1986). Croxall (1993) provides good identification sources for Antarctic prey.

Teleost fish are typically identified using sagittal otoliths. These are also are used to determine the size (length) of fish, allowing estimates to be made of prey mass using length–mass regressions (www.fishbase.org). Identification of fish species with fragile otoliths (e.g. salmonids, clupeids) or cartilaginous structures (e.g. elasmobranchs) can be improved/achieved using alternative diagnostic skeletal structures (e.g. vertebrae, jawbones, angulars, radials, otics, gill rakers, branchials, operculums, scutes, quadrates, teeth). The approach of using all hard parts is particularly useful when prey heads (i.e. otoliths) are not consumed, as sometimes observed for seals eating salmon. Lance *et al.* (2001) provided a list of key structures commonly used to identify and enumerate prey consumed in the North Pacific.

9.5.3 Prey enumeration using minimum number of individuals (MNI)

The minimum number of individuals (MNI; White 1953) is typically calculated to estimate the number of prey eaten from prey structures recovered. MNI attempts to avoid counting the same prey item more than once, by estimating the smallest number of individuals needed to account for the recovered structures of that taxon. Theoretically, MNI yields a better estimate of the actual number of individuals consumed when the meal consists of relatively few individuals (Nichol and Wild 1984; Joy *et al.* 2006).

Different methods of calculating MNI are needed because skeletal structures can be individual, paired, or multiple (see Lance *et al.* 2001). Fragments from broken structures are matched where possible. For paired unique structures like sagittal otoliths, it is simplest to assume that the number of fish is half the number of otoliths, except when sagittae look like other otolith pairs as in gobies. Refinements include determining separate left-, right-, and unknown sides, and calculating MNI as the greatest number of left or right elements (upper or lower beaks in the case of cephalopods). For species that vary greatly in size, the size range or type of the structure may be used to refine the MNI estimate. When using multiple structures, prey numbers are determined from the structure that yields the highest MNI. Direct counts can be used for individually occurring structures such as the atlas (first vertebrae) or vomer bones. Recovered vertebrae counts can be divided by the actual number of vertebrae recorded for that species (e.g. Ford 1937; Hart 1973; www.fishbase.com). Typically, for non-unique structures such as gill rakers, teeth, and fragments of cephalopod gladius ('pen'), an MNI of one is applied. Pens may be used to indicate the presence of squid, but can only be used for enumeration if they are intact (which is rare, given their fragility). The eye lenses of cephalopods are slightly flattened unlike fish

eye lenses which are spherical, and are useful elements to determine recent cephalopod ingestion (Staniland 2002). The number of individual crustaceans is based on eye, telson, or carapace counts.

9.5.4 Measurement of prey structures

Otoliths are the most widely used structure to determine fish size. Otolith length or width is typically recorded. Some authors have used otolith thickness, although this is generally more difficult to measure. In general, otoliths used for diet estimation refer to the sagittae, as they are usually bigger and their shapes show consistent interspecific variation. In the Gobiidae, lapillae otoliths are very similar to sagittae and so the number of gobies should be determined as a quarter of the number of otoliths. Unbroken otolith length should be measured to the nearest 0.1 mm (using digital callipers or a microscope and graticule), parallel to the sulcus, from the anterior tip of the rostrum to the posterior edge. Otolith width should be measured perpendicular to the sulcus at the widest point of the otolith, especially for those species with fragile post-rostrums (Fig. 9.1). Measurement protocols for other fish structures vary, but selection is typically based on those structures that are most robust to digestive processes (e.g. jawbones and vertebrae) and on the availability of regressions to convert the size of the measured structure to an estimate of prey size eaten (Watt *et al.* 1997; Zeppelin *et al.* 2004).

Random sub-sampling of prey items can be used when there are large numbers of a particular prey type. Appropriate sub-sample size will depend on the variability in the size of the measured structure and the degree of precision required (see Bowen and Harrison 1994).

For cephalopods, the lower beak is generally used for identification and measurement, although upper beaks can also be used, but far fewer regressions are available. Rostral length is normally measured in squid and cuttlefish and hood length in octopods (Figs 9.2a, b; Clarke 1986). Rostral width has been recommended for *Loligo opalescens*. The size of krill can be estimated from measurements of body length (anterior edge of the eye to the tip of the telson, excluding setae) or carapace length (tip of the rostrum to the mid-dorsal posterior edge) (Croxall 1993).

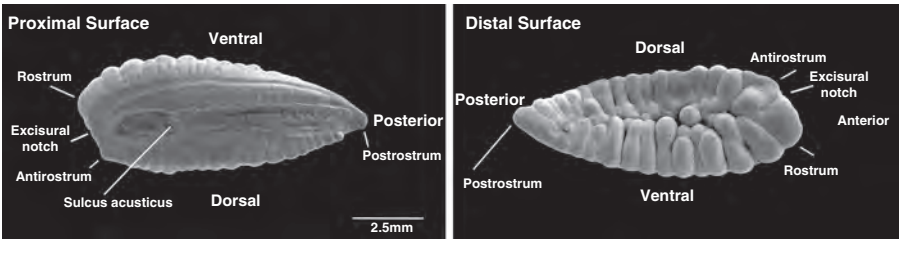

Fig. 9.1 Sagittal otolith from a teleost fish (family: Gadidae). (Photo courtesy of Dr Steven Campana, Bedford Institute of Oceanography, Canada.)

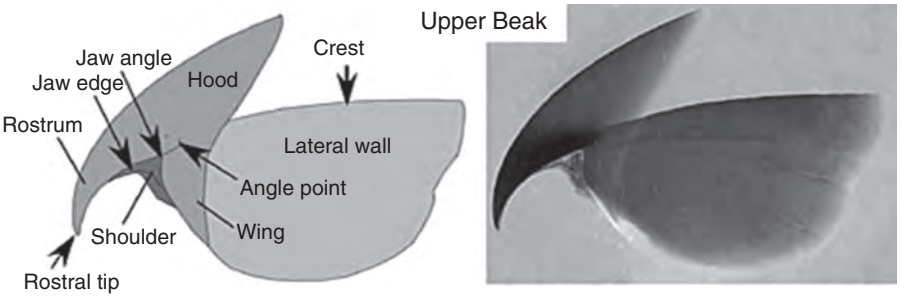

Fig. 9.2a Side views of the upper beak of *Stigmatoteuthis hoylei*. The rostral lengths should be measured from the tip of the rostrum to the jaw angle. Hood lengths should be measured from the tip of the rostrum to the tip of the hood. (Left, drawing courtesy of R. Young, University of Hawaii; Right, photo courtesy of R. Young.)

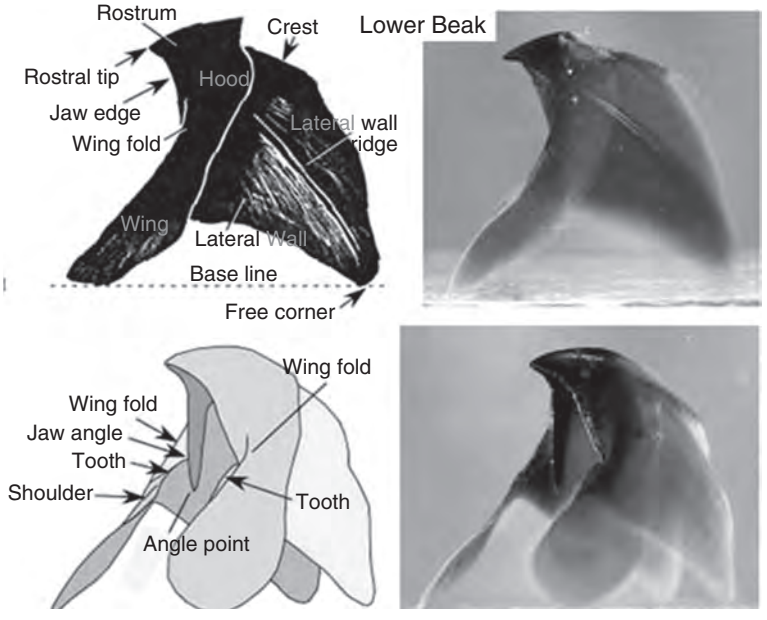

Fig. 9.2b Side views of the lower beak of *Stigmatoteuthis hoylei*. (Left, drawing courtesy of R. Young; Right, photo courtesy of R. Young.)

Separate regressions are often developed for each sex and reproductive status category, so these characteristics should be recorded when possible. Beaks can be measured using a microscope, digital camera and image measurement software (AxioVision 3.1) or using a binocular microscope equipped with an eyepiece graticule, or with Vernier calipers.

9.6 Quantification of diet composition using GI tract and faecal analyses

Biomass reconstruction using otoliths, beaks, and other hard part structures should account for structures that may be partially or completely eroded during digestion, as well as assessing levels of uncertainty (Bowen 2000; Hammond and Grellier 2005; Sigler *et al.* 2009).

9.6.1 Accounting for complete digestion of hard part structures

Numerical correction factors (NCFs) were introduced for pinniped faecal analysis (Harvey 1989) to take account of interspecific differences in otolith/beak recovery (the probability of passage), which had been shown to bias diet analyses in favour of species with large and robust hard parts (e.g. gadids, cephalopods) compared to smaller or more fragile prey with less robust structures (e.g. clupeids, scrombrids, salmonids, osmerids) (Prime 1979; Bigg and Fawcett 1985). NCFs are typically generated in captive feeding studies by comparing known numbers of prey consumed with estimates derived from reconstructing the number of prey, using MNI counts based on the structures that survive digestion (Harvey 1989; Bowen 2000; Tollit *et al.* 1997, 2007; Grellier and Hammond 2006). NCFs are applied to counts of prey before estimating prey mass. Fish otolith NCFs based on captive feeding studies typically range from 1.0 (where numbers fed are the same as the number estimated using MNI counts) to ~10.0 (i.e. only 10% of the numbers fed were recovered, as estimated from MNI counts). Relatively robust NCFs are available for key prey of just a few species of pinnipeds (notably grey seals, harbour seals, Antarctic fur seals (*Arctocephalus gazella*), Steller sea lions (*Eumatopias jubatus*), and California sea lions (*Zalophus californianus*). The application of NCFs is complicated by a range of extrinsic and intrinsic factors that may affect the values of these coefficients, such as meal size (Marcus *et al.* 1998), activity (Bowen 2000; Tollit *et al.* 2003), prey size (Tollit *et al.* 2007), study animal (Orr and Harvey 2001; Cottrell and Trites 2002), method of ingestion (Grellier and Hammond 2005) and the diet composition (Casper *et al.* 2006). Realistic NCFs are therefore hard to derive. Captive feeding studies using standard protocols are important to provide robust comparisons across prey species. Otolith robustness (pristine otolith length divided by otolith mass) is correlated with the probability of recovery in scats, but not in captive studies (see Harvey 1989; Tollit *et al.* 1997, 2007).

Differences in the recovery rates of prey species can also be reduced, but not eliminated, by using multiple structures rather than otoliths alone. The use of multiple structures is particularly useful for identifying and counting more fragile prey species (e.g. clupeids salmonids, and elasmobranchs). However, their use can also increase biases due to double counting prey across scats (Joy *et al.* 2006), unless counts for each species are based only on the skeletal element (of that species) which yields the highest count. Overall, while the application of NCFs (in captive

studies of pinnipeds at least) clearly improves the accuracy of dietary biomass estimates (see Tollit *et al.* 2007; Philips and Harvey 2009), the effect of applying NCFs in general will depend on the relative proportion of prey species in the diet and the NCFs of these species. In one field study, as an example, applying NCFs reduced the apparent importance of gadids in the diet by about 30–40% (Tollit, unpubl. data).

NCFs are currently unavailable for cetaceans. Most dietary studies on cetaceans are based on stomach contents, for which the degree of digestion will vary according to the time since ingestion. Murie (1987) suggested that the proportion of fish otoliths recovered inside fish skulls could be used to indicate the time since ingestion, and similar indices based on, for example, otolith degradation could be constructed. However, calibration of these indices, and derivation of NCFs for a range of times since ingestion, would require extensive experiments.

9.6.2 Other factors affecting recovery of hard part structures

Most remains of fish hard parts pass through the digestive tract between 1 and 3 days after ingestion (minimum 2 h, maximum 142 h; Harvey 1989, Fea and Harcourt 1997; Orr and Harvey 2001; Staniland 2002; Tollit *et al.* 2003); but interspecific rates can vary by a factor of two, and scats can represent a composite of up to six past meals (Tollit *et al.* 2003). Cephalopod beaks are far more resistant to the digestive process (Bigg and Fawcett 1985) and can be retained in the GI tract for up to three weeks (Tollit *et al.* 2003). Small beak recovery is higher than for large beaks, which may break or be regurgitated (Yonezaki *et al.* 2005). Consequently, the frequency of large cephalopods may be underestimated.

Australian sea lions (*Neophoca cinerea*) grind most identifiable hard parts into a paste, making hard part analysis unreliable (Gales and Cheal 1992; Casper *et al.* 2006). Captive feeding studies with otariids have shown that the remnants of meals containing relatively large fish as well as cephalopods can be regurgitated, so that structures from these prey will not appear in the scats. The collection of regurgitates is therefore important (see Gudmundson *et al.* 2006). Some otariids will also ingest gastroliths (stomach rocks), which likely affect the digestive process, recovery rates, and the identification of hard parts (Needham 1997; Tollit *et al.* 2003).

9.6.3 Accounting for partial digestion of hard part structures

Estimating the size of fish from measurements of otoliths (or other structures) recovered from scats and GI tracts may be biased because of partial digestion. For example, sizes of fish eaten by captive harbour seals were underestimated by 0–76% when back-calculated from otolith measurements (Tollit *et al.* 1997). To compensate for this bias, digestion coefficients or correction factors (DCFs) can be applied to otolith measurements (e.g. North *et al.* 1983; Harvey *et al.* 1989; Reid 1995; Tollit *et al.* 1997), and also to measurements of various other fish skeletal structures (Tollit *et al.* 2004). Larger otoliths are digested proportionally more than

smaller otoliths, meaning that care must be taken not to create bias by only selecting uneroded otoliths (Tollit *et al.* 1997; Grellier and Hammond 2006). Furthermore, the application of an average species-specific DCF may be unwise if there is high intraspecific variation in otolith digestion (Dellinger and Trillmich 1988; Tollit *et al.* 1997; Grellier and Hammond 2006).

Grade-specific DCFs are based on changes in the morphology and surface topography. Typically, structures are grouped into three or four different digestion grades (e.g. pristine, good, fair, poor), based on objective keys and photographic references (see Tollit *et al.* 1997; Leopold *et al.* 2001; Tollit *et al.* 2004; Grellier and Hammond 2006). Pristine graded structures are usually associated with regurgitations (or relatively undigested stomach contents) and therefore no correction is required. Structures classified in poor condition generally need not be measured (except perhaps to estimate a minimum size), as correction factors applied to otoliths in this condition result in wide confidence intervals around estimated prey lengths and weights (Tollit *et al.* 1997). Structures in 'good' condition are typically reduced 5–15% in size, while those in 'fair' condition are reduced 15–30% in size, though prey size is an additional variable (Tollit *et al.* 1997, 2004; Grellier and Hammond 2006). Grade-specific DCFs are best calculated from captive feeding studies and are applied before applying allometric body size regressions. In contrast to otoliths, in general there is little size reduction of cephalopod beaks (Harvey 1989) or crustacean carapaces (Staniland 2002).

9.6.4 Prey length and mass reconstruction

There are allometric relationships between otolith (or lower beak) size and fish (or cephalopod) size (see Casteel 1976; Härkönen 1986; Clarke 1986; Harvey *et al.* 2000). Fewer published relationships exist for other skeletal structures (but see Watt *et al.* 1997; Zeppelin *et al.* 2004). For most species, the relationship between otolith length and fish length can be described by a simple linear regression (sometimes with an inflection point), whereas conversion directly to mass is typically a power function.

Fish mass can be estimated directly from otolith length or by first estimating fish length. The two-stage procedure is particularly useful if information on seasonal variation in length–weight relationships is available, because the otolith–fish length relationship is more likely to be consistent across seasons. However, potentially, this approach leads to wider confidence limits (Casteel 1976). Most studies use linear regressions with log-transformation, but weighted non-linear regression is also used (Leopold *et al.* 2001).

Applying regressions introduces both random and systematic errors. The errors introduced by deriving fish weight from linear regression equations can readily be estimated using bootstrap techniques (Hammond and Rothery 1996; Pierce *et al.* 2007), provided that either the raw data or error parameters for the regression line

are available. Investigators should be cautious about using regressions based on collections from outside the study region, small sample sizes, or fish sizes outside the size range observed by the study in question (Harvey *et al.* 2000).

9.6.5 Quantification methods

To allow across-study comparisons, we recommend that authors report the total numbers of samples collected that contained (1) no prey remains (empty), (2) at least some identifiable prey remains, and (3) only prey remains that were 'unidentifiable' (hard parts too eroded for identification) or 'unidentified'. Regurgitations, stomach contents, and scats should be reported separately as they provide different representations of the diet.

The relative importance of prey in diets can be expressed in a variety of ways. Numerical counts of prey (e.g. percentage of the number of individuals for each prey taxon as a percentage of the total number of individuals found in all samples) is a simple method that is susceptible to overestimating the importance of small prey, mainly due to differences in the number of prey consumed per meal. Percent frequency of occurrence (percentage of samples containing a given prey taxon) is the simplest method to represent diet. It describes the number of animals eating a prey type and is probably least affected by interspecific differences in prey recovery. In general, occurrence indices may overestimate the importance of small or trace prey (especially when eaten in small numbers), while biomass indices may overestimate the importance of large prey. Volumetric indices are most useful for fresh stomach samples, while composite indices (such as index of relative importance, Pinkas *et al.* 1971), dominance, abundance, and diversity indices can be useful for comparative purposes (see Lance *et al.* 2001). The energetic contribution of prey to the predator's diet is probably the currency of most relevance to foraging animals. Detailed discussions of the advantages and disadvantages of the various measures of diet have been presented in reviews by Berg (1979), Hyslop (1980), Pierce and Boyle (1991), and Laake *et al.* (2002).

Numerical importance (N_i) for prey category i can be calculated as:

$$N_i = \frac{\sum_{k=1}^{s} n_{i,k}}{\sum_{i=1}^{\omega}\sum_{k=1}^{s} n_{i,k}} \text{ or } N_i = \frac{\sum_{k=1}^{s}\left[\frac{n_{i,k}}{\sum_{i=1}^{\omega} n_{i,k}}\right]}{s}$$

where ω = number of prey categories, s = number of samples, and $n_{i,k}$ is the number of individuals of the ith prey category in the kth sample. The first of these estimators is the more usual, in which the distribution of each prey category between scats is ignored and a single scat with a high number of a particular prey category can thus have a strong influence on the estimated overall importance of that prey category. In the second estimator, the contribution of each scat is effectively weighted equally.

How averages should be calculated depends on the nature of the samples and on the question asked (see Pierce *et al.* 2007 and p. 208 below).

Percentage frequency of occurrence (%FO), can be re-scaled so that, summed across all prey types, the values total 100%, and is then termed modified FO (MFO; Bigg and Perez 1985). Olesiuk *et al.* (1990b) proposed a split-sample FO (SSFO) estimator which examines species occurrence in each scat sample individually, apportioning the contribution of each prey category to each scat depending on the number of other species present, and assuming that each prey category present in each scat has been consumed in equal proportions. These two variants of the percentage FO method differ only in that MFO takes an equal weighting approach and SSFO an unequal weighting approach (see equations below). Olesiuk (1993) showed that when using SSFO the diet composition percentages for the primary prey varied by a factor of two or three, depending on the assumed composition within each scat. It is this composition of prey within a sample that biomass models aim to estimate.

$$FO_i = \sum_{k=1}^{s} I_{i,k} \; ; \; \%FO_i = 100 \times \frac{\sum_{k=1}^{s} I_{i,k}}{s} \; ; \; MFO_i = \frac{\sum_{k=1}^{s} I_{i,k}}{\sum_{i=1}^{\omega}\sum_{k=1}^{s} I_{i,k}} \; ; \; SSFO_i = \frac{\sum_{k=1}^{s}\left[\frac{I_{i,k}}{\sum_{i=1}^{\omega} I_{i,k}}\right]}{s}$$

where I = indicator function equal to 1 if the ith prey category is present in the kth sample, and 0 if it is absent.

Similarly, there are two approaches to estimating the weight (W) or the reconstructed biomass (BR) of prey consumed in the diet. Once again, each scat can be considered as contributing a variable amount (e.g. VBR) or a fixed (equal weighted) average amount (e.g. FBR).

$$W_i = \sum_{k=1}^{s}\sum_{j=1}^{n_{i,k}} w_{i,j,k} \text{ or } W_i = \sum_{k=1}^{s} n_{i,k} \times \bar{w}_{i,k} \; ;$$

$$VBR_i = \frac{\sum_{k=1}^{s} n_{i,k} \times \bar{w}_{i,k}}{\sum_{i=1}^{\omega}\sum_{k=1}^{s} n_{i,k} \times \bar{w}_{i,k}} \; ; \; FBR_i = \frac{\sum_{k=1}^{s}\left[\frac{n_{i,k}\times\bar{w}_{i,k}}{\sum_{i=1}^{\omega} n_{i,k}\times\bar{w}_{i,k}}\right]}{s}$$

where $n_{i,k}$ is the number of prey of category i in sample k, $w_{i,j,k}$ is the weight of the jth individual of prey category i in sample k, and $\bar{w}_{i,k}$ is the average weight of an individual of prey category i in sample k.

Most recent dietary studies have used the VBR (Hammond and Rothery 1996) rather than the FBR method, and the variable estimator also seems to perform marginally better in the analysis of captive feeding data (Tollit *et al.* 2007). Further work on foraging patterns is required to determine, in particular, if meal size actually varies systematically with prey type and availability (justifying VBR). Overall, given the assumption that a reconstructed biomass of scats does reflect variability in foraging success and meal size (consumption), we recommend applying the VBR method, coupled with the calculation of confidence intervals and an assessment of outliers. Choice of VBR assumes a negligible impact of other factors that likely influence reconstructed biomass, such as differences in digestion and subsequent deposition, and the inclusion of partial scat samples. Given these assumptions, comparison with FBR is considered worthwhile. The same weighting issue arises for pooling multiple collections.

Relatively little attention has been given to evaluating the uncertainty associated with estimates of the species composition of diets (Pierce and Boyle 1991; Tirasin and Jorgensen 1999; Hammond and Grellier 2005). To account for uncertainty due to sampling, non-parametric 95% confidence intervals (95% CI) for diet compositions can be generated by bootstrapping (Reynolds and Aebischer 1991). Bootstrap techniques can be also be applied to each stage of the quantification process, such as assessing errors around regressions or correction factors, provided that some information is available about the underlying uncertainty in each part of the calculations (Hammond and Rothery 1996; Tollit *et al.* 1997; Santos *et al.* 2001; Stenson and Hammill 2004; Pierce *et al.* 2007). Alternatively, variance estimators for diet composition can be constructed using finite population sampling methods (Cochran 1977) and delta method approximations based on the Taylor series (Seber 1973; Laake *et al.* 2002).

Given potential biases and often low precision, point values for prey proportions in a diet should be treated with appropriate caution (Hammond and Grellier 2005; Matthiopoulos *et al.* 2008). Diet studies need to address sampling and digestion-related biases, report confidence intervals, and highlight limitations. Consumption estimates are needed to estimate marine mammal predation of commercial fish species or of endangered prey (see Chapter 8). In addition to diet composition, accurate consumption estimates rely on good data on population size, age structure, and energetic requirements (Mohn and Bowen 1996; Shelton *et al.* 1997; Stenson *et al.* 1997; Winship and Trites 2003).

9.7 Molecular identification of prey remains

Protein electrophoresis and immunoassays using polyclonal antisera have been used with some success in detecting single species from GI tract and scat material. However, discerning mixtures of prey appears problematic and proteins degrade during digestion, reducing antigenicity (Pierce *et al.* 1990, 1993). An enzyme-linked immunosorbent assay (ELISA) enables rapid screening to obtain accurate data on gut contents (Sunderland 1988).

In contrast, there have been major advances in using DNA to study diets. This is based on the ability to identify unique pieces of DNA from either the predator (Reed *et al.* 1997) or the prey (Jarman *et al.* 2002; Casper *et al.* 2007a) species. There are two approaches to the identification of species-specific prey DNA sequences. The first technique involves polymerase chain reaction (PCR) amplification of prey DNA from tissue homogenates (e.g. stomach or scat samples) using group-specific primers (e.g. fish, cephalopods, krill; see Jarman *et al.* 2004; Deagle *et al.* 2005). To distinguish the different sequences (species) represented, the amplified DNA is then analysed using a technique such as high-resolution gel/capillary separation, DNA cloning, or restriction fragment length polymorphism. The DNA can then be sequenced. Sequences can then be matched to species using basic local alignment search tool (BLAST) database searches. The second approach involves the amplification of prey DNA using species-specific targets (Jarman *et al.* 2002; Casper *et al.* 2007a). This has become popular because it is relatively simple and inexpensive to design PCR primer sets that target organisms at various taxonomic levels. Group-specific DNA primers allow an even broader survey of prey types to be conducted (see King *et al.* 2008). These PCR-based techniques have been applied to DNA extracted from scat and stomach remains, as well as prey remnants collected after surface feeding events. Reed *et al.* (1997) were also able to identify individuals, species (pinniped), and the sex of seals at mixed-species haul-outs. PCR techniques were used to identify different species of salmon from bone fragments recovered from seal scats (Purcell *et al.* 2004; Parsons *et al.* 2005) and also using fish scales collected after killer whale feeding events (Ford and Ellis 2006).

Tollit *et al.* (2009) used group-specific nested primers followed by denaturing gradient gel electrophoresis (DGGE), a technique which separates amplification products based on their melting behaviour as they denature, to detect >40 different species from the scats of Steller sea lions. DNA identification increased the number of prey species detections by 22% compared with conventional morphological identification. Captive feeding studies with sea lions have shown that the detection of prey in faecal matter is limited to those consumed during a 48-h period before defecation (Deagle *et al.* 2005), suggesting analysis of prey flesh may be more representative of recent feeding and not a composite of meals from many days.

To date, conventional PCR has provided only occurrence data rather than quantitative estimates of the proportion of each prey eaten. Consequently, quantitative real-time PCR (qPCR) methods, which measure the amount of DNA by fluorescence monitoring of PCR, have been performed with seal scats and these deserve further development. To date, qPCR studies highlight high detection sensitivity (0.01%) and the potential to estimate relative quantities of a target species, but there is a need to assess prey DNA degradation during digestion in mixed-prey species studies (Deagle and Tollit 2007; Matejusová *et al.* 2008).

Potentially, biases equivalent to those that affect quantitative estimates of diet from hard parts will have to be overcome. Due to development costs, qPCR methods are presently likely to be limited to very specific questions, such as the contribution of salmonids in a predator's diet. As DNA mass target detection systems improve and become less costly, multiplex PCR, microarrays, and, in particular, pyrosequencing appear to have great potential in the future (see review in King *et al.* 2008; Deagle *et al.* 2009; Dunshea 2009).

Finally, near infrared spectroscopy (NIRS) is relatively new. It estimates the composition of an organic sample when it is irradiated with light in the NIR spectrum. Organic material absorbs NIR light at wavelengths characteristic of particular bonds. Therefore, the amount of light reflected at a given wavelength is indicative of the concentration of compounds with that particular bond. Kaneko and Lawler (2006) tested the method using scats from captive otariids that were fed mixed-species meals, and this study represents the only ever test. In five of six cases, NIRS accurately and precisely quantified how much of a given diet component a seal had eaten the previous day. The authors suggest the technique may be especially useful in cases where there are particular, or few, prey species of interest.

9.8 Fatty acid (FA) signatures

Fatty acids (FAs) are the largest constituent of neutral lipids, such as triacylglycerols (TAG) and wax esters (WE), as well as of the polar phospholipids (PL). All FAs consist of carbon atom chains, which are most commonly even-numbered in length and straight, containing 14–24 carbons and 0–6 double bonds with a methyl (CH_3) terminal at one end and an acid (carboxyl, COOH) group at the other (see reviews of Dalsgaard *et al.* 2003; Budge *et al.* 2006; Iverson 2009b). FAs of carbon chain-length 14 or greater pass into the circulation intact and are generally taken up by tissues the same way. Although some metabolism of FAs occurs within the predator, such that the composition of predator tissue will never exactly match that of their prey, many FAs can be deposited in adipose tissue with relatively little modification and often in a predictable way (Iverson *et al.* 2004; Iverson 2009b).

Three characteristics of FAs and their storage permit them to be used as trophic tracers. First, since a relatively limited number of FAs can be biosynthesized by animals, especially at higher trophic levels (Cook 1996), it is possible to distinguish dietary versus non-dietary sources. Second, unlike proteins and carbohydrates, which are completely broken down during digestion, FAs are generally not degraded during digestion and are taken up by tissues in their original form. Third, fat is stored in animal bodies in reservoirs, which can be substantial. Thus, FAs accumulate in storage sites over time and represent an integration of dietary intake over days, weeks, or months, depending on the species and its energy intake and storage rates (Iverson 2009b).

It has been known for decades (e.g. Klem 1935) that FAs are transferred from prey to predator both at the bottom and top of marine food webs (reviewed in Dalsgaard *et al.* 2003; Budge *et al.* 2006; Iverson 2009b), permitting inferences about consumer diets. FAs can be used to study diets in marine mammals in three ways. First, by examining changes in FA distributions, or 'signatures' (Iverson 1993) of the predator alone, qualitative questions about spatial or temporal variations in diets can be addressed, both among and within individuals or populations (e.g. Iverson *et al.* 1997a, b; Walton *et al.* 2000; Beck *et al.* 2005). Second, the presence or abundance of unusual FAs in predator tissues can be traced to the consumption of a particular prey species or taxa (e.g. Pascal and Ackman 1976; Thiemann *et al.* 2007). Third, and requiring the most careful considerations, FAs can used to quantitatively estimate diet from the FA signatures of predators using quantitative FA signature analysis (QFASA, Iverson *et al.* 2004), which employs a statistical model to compute the likely combination of prey FA signatures that comes closest to matching that observed in the predator FA storage sites (Iverson *et al.* 2004, 2006, 2007).

9.8.1 Tissues for analysis

Predators

Adipose tissue (including blubber), milk, and blood, contain the most direct information about diet (Budge *et al.* 2006; Iverson 2009b). The more membrane-structured the tissue (e.g. structural blubber such as tailstocks in cetaceans and muscle), the greater the contribution from endogenously conserved FAs which may obscure dietary influences to lesser or greater extents. Selection of tissue type will depend on the research question and sampling limitations of the species. Both adipose tissue stores and non-structural blubber contain an integrated record of dietary intake over a period of weeks to months, and perhaps longer in some species. However, the time frame of integration has only been investigated in a few species and thus more research is needed. The FA composition of milk also reflects diet, but the temporal nature of this information depends on the reproductive strategy of the particular species. In capital breeders, such as phocid seals and many baleen whales, milk FAs will be derived from blubber mobilization and thus reflect diet over the months of fattening prior to lactation. Conversely, in income breeders (e.g. otariids, small odontocetes), milk FAs will reflect the most recent dietary intake (i.e. days), as well as some mobilization of FAs from fat depots. Quantitative diet estimates from milk FAs using QFASA have not yet been attempted or validated. Finally, FAs can also be isolated from blood in the form of chylomicrons, which are the lipoproteins that specifically carry FAs from recent digestion throughout the bloodstream. Chylomicrons only persist in the blood for 2–6 hours, but when correctly isolated from blood, have provided accurate estimates of the most recent meal in grey seals (Cooper *et al.* 2005).

Subcutaneous adipose tissue/blubber can be sampled easily from live animals using a medical biopsy punch (e.g. 6 mm diameter), normally inserted through a small incision made in the skin through the full depth of the blubber layer (e.g. Kirsch *et al.* 2000). In most situations, it is considered best to leave the incision open, rather than suturing it closed, for the best draining and healing. Samples are easily obtained from dead animals by incision, but should be taken as soon as possible after death and certainly within 24 h of death. The FA composition of true fat storage sites (i.e. subcutaneous adipose tissue, non-structural blubber) appears to be homogeneous over most of the main body in many species (Koopman *et al.* 1996; Layton *et al.* 2000; Cooper 2004; Thiemann *et al.* 2006; Iverson *et al.* 2007). Although this should be confirmed in each species, it appears that the best site for sampling subcutaneous adipose tissue is simply where most fat is normally and actively stored. Of greater importance for blubber is the depth sampled (e.g. Koopman *et al.* 1996; Arnould *et al.* 2005; Strandberg *et al.* 2008). In pinnipeds, blubber is not 'stratified' per se, but there exists a gradation in FA composition from the inner to outer portions. Studies have shown that the full-depth blubber layer provides information on longer term diet, while the inner half alone reflects a more recent diet (Cooper 2004; Iverson *et al.* 2004; Tollit and Iverson, unpubl. data). Small samples only taken near the skin are not appropriate for diet assessment (Thiemann *et al.* 2004, 2009). In contrast to pinnipeds, the blubber of cetaceans, particularly small odontocetes, can exhibit extensive morphological and FA stratification, but the degree to which this occurs is species-specific (Koopman 2007). In some cases, FA stratification is sufficiently extreme that only the innermost layer can be used to infer diet, as the FAs stored in the outer layers appear to be largely endogenously derived and conserved. However, FA stratification appears to be less pronounced in larger cetacean species such as the bottlenose whale (*Hyperoodon ampullatus*) (Hooker *et al.* 2001a) and sperm whale (Koopman 2007).

Milk for FA analysis can be obtained directly from evacuation of mammary glands or taken from the stomach contents of neonates up to 8 h following ingestion, as the FA composition remains unchanged within the gastric milk fat globule until it is disrupted in the intestine (Iverson 1988).

Blood may also be used for FA analysis, but because of the ephemeral nature of dietary lipid transport in blood, the time of collection with respect to feeding is critical. It is essential that the TAG-rich chylomicrons carrying dietary FAs are: (i) visibly present in samples (as indicated by a cloudy or milky hue) and (ii) isolated from other blood lipids and lipoproteins (Cooper *et al.* 2005), as analysis of whole blood, plasma, or serum will lead to highly erroneous diet inference. The isolation of chylomicrons from other lipoprotein classes is performed by ultra-centrifugation of freshly collected serum or plasma samples (both give identical results). Even short-term freezing may cause disruption of the chylomicron lipids.

Prey

If FAs are to be used to estimate diet quantitatively, then prey must be sampled as has been extensively reviewed elsewhere (Iverson *et al.* 2004; Budge *et al.* 2006; Iverson 2009b). In brief, the onus is on the investigator to reasonably sample the prey field of the predator, to sample prey in the same manner in which the predator consumes these prey (i.e. for most marine mammals prey is consumed whole), and to sample a sufficient number of individuals of each prey species to allow assessment of within-species variability and between-species differences or overlap (Budge *et al.* 2002; Iverson *et al.* 2002, 2004). Prior to chemical analysis, each individual should be measured (mass, length), homogenized, and a weighed aliquot taken for analysis of fat content and FA composition. A thorough quantitative evaluation of within- and between-species variability to confirm the ability to reliably differentiate prey species in the estimation procedures is required.

9.8.2 Sample storage and chemical analysis

Guidelines for the optimal storage of tissues for lipid and FA analysis are summarized in Budge *et al.* (2006). Exposure to air will oxidize FAs in the sample, with a loss of especially highly unsaturated FAs. Freezing at −80 °C in an airtight container is one recommendation for long-term storage, but the lipids in the sample can be preserved indefinitely by immediately immersing the sample in chloroform ($CHCl_3$) containing 0.01% butylated hydroxytoluene (BHT) as antioxidant (however, note the biohazard issue with using chloroform). If frozen at −20 °C, especially under a nitrogen atmosphere, such samples may be safely stored for years. Only glass vials with Teflon-lined caps can be used for FA storage in solvents, as $CHCl_3$ will extract the plasticizers in other types of containers and contaminate the isolated lipids.

Methods for extraction from tissues, preparation, and analysis of FAs have been extensively reviewed (Christie 1982; Ackman 1986, 2002; Parrish 1999; Iverson *et al.* 2001; Budge *et al.* 2006). Briefly, lipids are best extracted with a modified Folch *et al.* (1957) procedure employing $CHCl_3$ and methanol (MeOH) (Iverson *et al.* 2001), which also allows quantification of fat content (see 9.8.4). Once extracted, acyl lipids are *trans*-esterified (i.e. converted to FA methyl esters, FAME, or FA butyl esters, FABE), ideally using an acidic catalyst, such as sulphuric acid (H_2SO_4) or hydrochloric acid (HCl) in methanol or butynol, respectively. Comparable results can be obtained using fresh anhydrous BF_3 and H_2SO_4 catalysts (Iverson *et al.* 1997a; Thiemann *et al.* 2004). However, anhydrous BF_3 in MeOH is no longer guaranteed by chemical suppliers, therefore we now recommend the use of the H_2SO_4 catalyst. FABE are only required in the case of some odontocetes that produce very short-chain volatile FAs, which are otherwise lost when using FAME.

Analyses of FA composition are performed using temperature-programmed gas chromatography (see methods in Iverson *et al.* 1997b, 2002; Budge *et al.* 2002). It is crucial to select a polar capillary column that allows adequate separation of all

peaks of interest, such as the many isomers of long-chain poly-unsaturated fatty acids (PUFA) and some mono-unsaturated fatty acids (MUFA) (e.g. *n*-11 and *n*-9 isomers of 20:1 and 22:1). Excellent separations of marine FAs are achieved using a very polar column coated with 50% cyanopropyl-methylpolysiloxane, specifically the DB-23 model from Agilent Technologies, but other columns can be used. FAs 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, given the complexity and number of marine FAs; therefore, chromatogram IDs and separations should be rigorously checked and reintegrated where necessary (Budge *et al.* 2006).

9.8.3 Using predator FAs to qualitatively infer diet

Evaluating variation in the full array of FAs (FA signatures) among individuals and populations of marine mammals is a promising, qualitative, way to look at trophic interactions and to detect dietary differences (S. Smith *et al.* 1997; Iverson *et al.* 1997a, b; Dahl *et al.* 2000; Thiemann *et al.* 2008; Tucker *et al.* 2009). Because over 70 different FAs are routinely identified in marine lipids, multivariate statistical techniques are generally required to best use the information contained in the data, although visual graphical inspection allows assessment of absolute differences. That is, finding a 'significant' difference in the levels of a specific FA among groups does not indicate whether this difference is biologically meaningful or whether the overall FA signature differs between groups. Multivariate analyses, which also allow pattern recognition, are generally the most powerful as they use the maximum number of FAs (depending on sample size) for differentiating predators and resolving trophic interactions (reviewed in Budge *et al.* 2006; Iverson 2009b).

The use of a FA 'biomarker approach' (i.e. using a single or group of unusual FAs or an unusual abundance of a FA to infer a predator diet item) will, in principle, rarely be possible in marine mammals given that they generally occupy the top of the food chain, and thus integrate consumption over several trophic levels. Although this has been done with some degree of success in several instances within simple systems, since it is generally a risky practice to infer diets directly from one or a few FAs, we point to reviews of this subject (Budge *et al.* 2006; Iverson 2009b).

9.8.4 Using predator and prey FAs to quantitatively estimate diet

QFASA is a first-generation statistical tool designed to quantitatively estimate predator diet using FA signatures of predator and prey. The basic approach of QFASA is to determine the mixture of those prey species FA signatures that most closely resembles that of the predator's FA stores, after accounting for the effects of predator metabolism, to thereby infer its diet. Details of the initial QFASA approach are provided by Iverson *et al.* (2004) and further discussed in subsequent studies and reviews (Budge *et al.* 2006; Hoberecht 2006; Iverson *et al.* 2006, 2007;

Beck *et al.* 2007; Nordstrom *et al.* 2008; Thiemann *et al.* 2008; Iverson 2009b; Tucker *et al.* 2009).

Briefly, QFASA proceeds by applying experimentally derived weighting factors ('calibration coefficients', CCs) to individual predator FAs to account for the effects of predator metabolism on FA deposition. It then takes the average FA signature of each prey species (or group), and estimates the mixture of prey signatures that comes closest to matching that of the weighted predator's FA stores by minimizing the statistical distance between that prey species mixture and the weighted predator FA profile. Lastly, this proportional mixture is weighted by the fat content (i.e. relative FA contribution) of each prey species to estimate the proportions in the predator's diet.

A number of important issues must be recognized in order to predict diet using QFASA (see Iverson *et al.* 2004; Budge *et al.* 2006; Iverson 2009b for detailed discussions). Perhaps the most important issue is that of accounting for predator metabolism (e.g. Nordstrom *et al.* 2008; Meynier 2009). At present, calibration coefficients are used. These are simple ratios for each FA in the predator stores divided by the level of that FA in the diet consumed over a period long enough for complete FA turnover. In principle, the FA signature of the predator's lipid stores should resemble this diet as much as possible and any differences would be attributable to metabolic processing of individual FAs. Although CCs are currently the only method put forward to account for predator metabolism, and they have been shown to result in reasonably accurate estimates of core diet, they remain a simple mathematical attempt to describe potentially complex biochemistry. Individual FA deposition could be affected by a number of factors, including physiological status (e.g. lactation, starvation) or external factors (e.g. prey fat content and possibly FA composition).

CCs have been estimated for a handful of pinniped species fed long-term on herring and, while many CCs are similar among these studies, recent validation studies suggest that predator species-specific CCs should be used if possible (e.g. Hoberecht 2006; Nordstrom *et al.* 2008). Whether these are truly species effects or diet/study effects are not yet known. Cetacean CCs have been harder to generate, mainly because of the requirement to sample full-depth blubber in captive animals that are on public display. In captive Steller sea lions, some dietary-related CCs varied with diet composition (Tollit, unpublished data). The application of these CCs in the QFASA model has been shown to be critical to optimal diet estimates (Iverson *et al.* 2004) and further studies of CCs are critical to further development.

Another issue in QFASA is which FA subset to use. Not all FAs provide information on diet (e.g. some arise in predators solely from biosynthesis), and those FAs that do not should be removed from analysis. Other FAs may be affected by the reliability of the CC calculated for them. Furthermore, not all FAs identified as useful and of dietary origin in the original model (Iverson *et al.* 2004), can be consistently and reliably detected by some laboratories, depending on

the equipment used (e.g. Hoberecht 2006). The choice of FAs may also affect how well each subset selection discriminates between different prey species. Subset selection should be thoroughly tested, and using captive validation studies when possible. Similarly, further studies are required to ascertain FA turnover time (normally approx. 1–6 months) and the potential interaction with life history events.

Simulation is a useful way to explore prey differentiation using QFASA (e.g. Iverson *et al.* 2004, 2006; Tucker *et al.* 2009). Understanding the detection limits of prey, the fitting procedures of statistical models (Stewart 2005), and inclusion of within-species variability in prey FA and fat content in estimates require further research.

To date, QFASA has been able to provide reasonably accurate estimates of simple diets and diet switches in captive pinnipeds (e.g. Cooper 2004; Iverson *et al.* 2004; Nordstrom *et al.* 2008). However, there may be more inconsistent estimates and increased rates of misclassifications if the diet is diverse, measured over a very short time (<10 days) or if it consists of multiple species with similar FA signatures (e.g. Hoberecht 2006; Tollit and Iverson, unpubl. data). Nevertheless, natural relatively complex diets have been estimated using QFASA for free-ranging seals and seabirds, which were corroborated using either animal-borne video or other methods of diet analysis (e.g. Iverson *et al.* 2004, 2007; Tucker *et al.* 2008).

9.9 Stable isotopes and other markers

Elements in nature typically occur in more than one stable form due to differences in atomic mass. Small differences in the mass of these stable isotopes mean they behave differently in biogeochemical reactions. In kinetic and other rate-limiting processes, the relative abundance of heavier to lighter isotopes can change among reservoirs within the body of an animal. Isotopic distributions in nature provide the basis for tracing the origins of elements and molecules spatially and through trophic interactions. The most common elements used in the study of marine mammals have been H, C, N, O, and S. These light elements are typically fixed during primary production and consist of 2–3 stable isotope forms: ^{1}H, ^{2}H, ^{12}C, ^{13}C, ^{14}N, ^{15}N, ^{16}O, ^{17}O, ^{18}O, ^{32}S, ^{34}S.

Measurements of slight differences in the relative abundance of one isotope over the other are possible through established mass spectrometric techniques. These usually involve the comparison of the relative abundance of the heavier to the lighter isotope of an element in unknown samples to those in international standards. This is the basis of the delta notation in isotopic measurements that represents a ratio of ratios:

$$\delta X = [\text{Rsample}/\text{Rstandard} - 1] \times 1000$$

where X is the heavy isotope of interest (e.g. ^{13}C, ^{15}N, ^{34}S) and R is the ratio of the heavy to light isotope (e.g. $^{12}C/^{13}C$, $^{14}N/^{15}N$, $^{32}S/^{34}S$). The international standards

are relatively arbitrary and so delta values can be negative (e.g. $\delta^{13}C$ values) or positive (e.g. $\delta^{34}S$ values). Units of measurement are parts per thousand ‰. Measurement error typically ranges from ± 0.1 ‰ for $\delta^{13}C$ to ± 2 for $\delta^{2}H$.

There are three primary concepts in the application of stable isotope measurements to marine mammal dietary studies. The first is that stable isotope values in food webs are largely determined by those values in inorganic substrates. The process of fixation of elements during primary production involves isotopic discrimination, whereby one isotope is differentially incorporated. Once fixed, elements move through food webs, and the measurement of stable isotope ratios in consumers can give some idea of the source of primary production in cases where sources may differ in their stable isotope values. The second concept is that of isotopic discrimination between prey and predator, with more or less consistent changes in isotope ratios as one ascends the food chain. However, not all tissues are created equally, and isotopic discrimination between diet and predator tissues can vary according to tissue type, diet quality, and differential metabolic routing of macromolecules within the organism. Finally, the turnover of elements can differ between tissues and the half-life ranges—from a matter of days in the case of blood plasma to months in the case of muscle, and even years in the case of bone collagen. Dietary information will thus be integrated over different time spans depending on the tissue used.

Dietary reconstructions in marine mammals require fundamental knowledge of the baseline isotope values in the food webs of interest, the isotopic discrimination factor between diet and a specific tissue, likely metabolic routing of macromolecules, and the turnover rate associated with the tissue sampled. There are few cases where such knowledge will be complete. The main limitation of stable isotope analysis is that it provides relatively coarse information on diet, since the contributions of all prey are reduced to a single ratio for each isotope and the small number of variables inevitably limits discrimination of diet composition. Nonetheless, there are some advantages to using stable isotopes in trophic studies involving marine mammals, and also in conjunction with other methods such as FA and QFASA (Budge *et al.* 2008; Tucker *et al.* 2008). Both methods provide information on food assimilated and integrated over a relatively long time span.

9.9.1 Tissues for analysis

Skin, blubber, teeth, baleen, and internal organs have been used from stranded animals, and whiskers, muscle, and blood components from sedated animals. At sea, remotely sampled biopsy plugs of skin and blubber are possible, as is the use of sloughed skin (Todd *et al.* 1997; Ruiz-Cooley *et al.* 2004).

Although diet-tissue discrimination factors are still poorly understood, they do allow dietary isotopic values to be predicted and to understand the period of dietary integration. However, some controlled captive studies of phocid (Hobson *et al.* 1996) and otariid (Kurle 2002) seals have helped derive approximations for various

blood components and whiskers (Hall-Aspland *et al.* 2005). The average transit time of dermal cells from the basal lamina to the skin surface, where they are sloughed, corresponds to about 75 days in the bottlenose dolphin (*Tursiops truncatus*) and beluga (*Delphinapterus leucas*) (Hicks *et al.* 1985; St. Aubin *et al.* 1990), but is probably longer for larger cetaceans. So, skin biopsies probably integrate isotopic information on diet for at least that period. For metabolically inactive tissues like baleen, nail, or whiskers, stable isotope values are typically unchanged following formation and so provide a temporal record of past diets (Schell *et al.* 1989).

Teeth contain both organic (i.e. collagen) and inorganic fractions in the dentine and enamel (Clementz and Koch 2004) and can be sub-sampled using the internal growth layers, or growth layer groups (GLG, see Chapter 5). The organic fraction can be analysed for all the light elements, and the inorganic fraction for $\delta^{13}C$ and $\delta^{18}O$ values. Age-specific trophic level estimates can be based on analysis of individual annuli (Hobson and Sease 1998; Hobson 2004a; Hanson *et al.* 2009). Mendes *et al.* (2007) inferred changes in trophic level and migration patterns in sperm whales from isotope measurements of tooth annuli.

9.9.2 Trophic modelling

Stable nitrogen isotope values ($\delta^{15}N$) in marine consumers show a step-wise increase with trophic level. Thus, by knowing the magnitude of this trophic enrichment corresponding to the tissue sampled, it is possible to predict (by subtraction) the average $\delta^{15}N$ value of the prey or, if the baseline food web $\delta^{15}N$ value is known, to model the trophic position of the organism of interest (e.g. Hobson and Welch 1992). Meta-analyses have suggested a $\delta^{15}N$ trophic enrichment factor for marine mammals of about 3.4 ‰ (Post 2002).

In many cases, the appropriate stable isotope values for primary production are poorly known, or are known to differ seasonally or over the large spatial areas used by many marine mammals. In such cases, it may be better to use $\delta^{15}N$ measurements of higher trophic-level (TL) primary herbivores (i.e. assumed to be at TL 2) as a baseline (Hobson *et al.* 2002; Hooker *et al.* 2002c; Ruiz-Cooley *et al.* 2004). Another consideration in applying $\delta^{15}N$ measurements to derive estimates of trophic level is that the values are averages. So, if two trophic sources were consumed in equal proportions by a consumer, then the intermediate trophic level predicted by the use of this single isotope measurement (i.e. [TL1 + TL2]/2) would be identical to that of another consumer feeding exclusively at that intermediate trophic level. Thus, it is essential to be aware of the inherent ambiguity in trophic models.

Theoretically, the transfer of maternal-based nutrients to offspring via suckling represents a trophic increase in the position of the neonate relative to the mother. Such a trophic increase should be reflected in higher $\delta^{15}N$ values in the offspring compared to the nursing parent (but see Jenkins *et al.* 2001). Indeed, this $\delta^{15}N$ enrichment effect has been observed in the first annulus of seal teeth (Hobson and

Sease 1998; Hobson *et al.* 2004a; Newsome *et al.* 2006, Hanson *et al.* 2009). A complementary decrease in dentine $\delta^{13}C$ values corresponding to the pre-weaning period also corresponds well with a neonate diet rich in ^{13}C-depleted lipids, since lipid carbon in milk contributes to protein synthesis in offspring. York *et al.* (2008) used $\delta^{15}N$ and $\delta^{13}C$ measurements of archived tooth annuli of Steller sea lions to infer how age of weaning was influenced by large-scale oceanic change in the Gulf of Alaska during the 1970s.

9.9.3 Source of feeding and marine isoscapes

Stable isotope analyses also provide the possibility of delineating spatial information on where marine mammals fed. Stable carbon isotope measurements are known to provide spatial information by latitude (Rau *et al.* 1982; Hobson *et al.* 1997a; Cherel *et al.* 2005). Off-shelf, pelagic food may also be more depleted in ^{13}C compared to on-shelf or benthic food, probably partly because of the depletion of $\delta^{13}C$ values of primary production in low nutrient conditions (Laws *et al.* 1995; France 1995). There is a longitudinal gradient in $\delta^{13}C$ values in the marine food webs along the northern Gulf of Alaska which can be detected in the tissues of Steller sea lions (Kurle and Gudmundson 2007). In addition to those of C and N, stable isotopes of other elements including S, O, and H have considerable potential to provide useful marine isoscapes. For example, $\delta^{34}S$ values may also be more enriched in benthic or inshore vs. pelagic food webs, and both δD (deuterium) and $\delta^{18}O$ are sensitive to salinity and ocean temperature (Hobson *et al.* 2010).

9.9.4 Other elements and compounds

Concentrations of other elements and compounds, especially inorganic and organic contaminants, in the tissues of marine mammals may provide information on diet and feeding provenance. Numerous studies have found good correlation between $\delta^{15}N$ values and contaminant loads in marine organisms (Atwell *et al.* 1998; Das *et al.* 2000; Fisk *et al.* 2001). As such, contaminant measurements themselves can be used to infer trophic level as well as geographical segregation in some systems (Shao *et al.* 2004). Recent advances in our understanding of strontium isotope ratios, and how these can influence tissue values in animals influenced by estuaries or terrestrial runoff, suggest this heavier isotope will be of use (Hobson *et al.* 2010).

9.9.5 Field and laboratory methods and data analysis

Tissues for stable isotope measurements should be frozen following collection. However, short-term storage of soft tissues in 70% ethanol has negligible effects on stable isotope measurements (Hobson *et al.* 1997b). Oven-drying may be used, but temperatures should not exceed 60 °C. Hard tissues like fur, whiskers, claws, teeth, and baleen can be stored dry. Hobson and Sease (1998) and Newsome *et al.* (2007) describe methods for isotopic analysis of the organic and inorganic fractions of teeth. Bone collagen extraction methods are found in Newsome *et al.* (2006).

Tissues are typically cleaned of surface contaminants before drying and homogenizing to a fine powder. For hard keratinous tissues, oils can be removed using a variety of solvents, but a 2 : 1 chloroform : methanol solution works well. Sonication can assist in surface cleansing. For soft tissues, following thawing or decanting of ethanol, freeze-drying is the preferred next step as this renders materials easy to powder by mortar and pestle or by mechanical grinders or mills. It is essential that the material analysed is homogenous.

Lipids are considerably more depleted in ^{13}C than most other animal tissues and, because they occur in varying concentrations depending on nutritional considerations, are best removed from bulk tissues such as muscle, liver, and blood in order to remove this source of variation on the tissue $\delta^{13}C$ value. This can be achieved using various techniques including soxhlet extractions or quicker solvent rinses on small powdered materials (Hobson *et al.* 2002). However, lipid extraction has also been shown to have a small but measurable effect on tissue $\delta^{15}N$ values. A good discussion on how to approach marine food web tissue analyses is provided in Søreide *et al.* (2007).

About 1 mg is required for most powdered samples of $\delta^{13}C$, $\delta^{15}N$ and $\delta^{34}S$, but this can vary depending on the elemental concentrations, and all samples must be weighed precisely (± 0.1mg). Continuous-flow, isotope-ratio, mass spectrometry (CFIRMS) is used almost universally these days. Researchers should contact appropriate labs to determine precise protocols and seek out labs that use *organic* laboratory standards (that are of a similar C : N ratio to their unknowns) when measuring organic materials. Researchers are also encouraged to provide blind replicates of their samples, as it is the replicate measurement of lab standards run with the samples or on a series of the same unknown that provides the measurement error that should be quoted (Jardine and Cunjak 2005).

It is common to portray results as biplots using two isotope measurements: most typically with $\delta^{13}C$ values on the x-axis and $\delta^{15}N$ values on the y-axis. Because different tissues can involve different isotopic discrimination values for a consumer, authors should either only include plots with the same consumer tissues represented or normalize all tissues to their diet equivalents by applying appropriate tissue-specific discrimination values. Other common uses of stable isotope data are to reconstruct dietary inputs to consumers using mixing models. The rule-of-thumb is that inputs from n sources of isotopically distinct foods can be uniquely resolved using n-1 stable isotopes (Phillips 2001). In cases of too many sources, ranges of inputs from specific sources can use probabilistic models (Phillips and Gregg 2003).

The successful application of stable isotope techniques for inferring the diet and source of feeding in marine mammals will depend on how well the researcher is able to characterize isotopically the food web being used. As such, an isotopic assay of prey and consumer tissues is encouraged over measurements of the consumer alone. Choice of tissue and knowledge of the period of dietary integration represented

by the isotope values is also critical. Researchers are encouraged to consider the use of more than one tissue in order to gain insight into diet over different periods for the same animal. This is a rapidly advancing field, and we anticipate more refined analyses being possible through the description of marine isoscapes on the one hand and the careful use of controlled dietary studies on captive animals on the other. Finally, stable isotope methods will typically augment, but not replace, the other tools we have to investigate marine mammal diet.

9.10 Summary

The emergence of various new techniques described in this chapter underlines the great potential to use multiple dietary assays. Ultimately, no single technique will provide all the answers, and researchers should aim to use as many lines of evidence as possible when weighing the evidence for marine mammal dietary compositions, especially if the species under study is considered a wide-ranging generalist consuming many different taxa. Stomach, regurgitate, or scat hard part analysis provides vital definitive species identification (as well as size) and should therefore form the baseline of new dietary studies, assuming samples are readily available. Many of the key limitations of hard part analysis are now well understood. The concurrent use of DNA methods on soft and hard prey remains in scats and GI tracts shows great promise and the field is developing quickly. FAs and isotopes typically provide less direct evidence of diet (and lower resolution in the case of stable isotopes - i.e. identifying trophic level) but importantly, over longer periods and samples can be collected directly from individual animals. This makes them attractive alternatives for many species of cetacean and wide-ranging pinnipeds.