



## Methods of estimating marine mammal diets: A review of validation experiments and sources of bias and uncertainty

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### ABSTRACT

Diet estimation in marine mammals relies on indirect methods including recovery of prey hard parts from stomachs and feces, quantitative fatty acid signature analysis (QFASA), stable isotope mixing models, and identification of prey DNA in stomach contents and feces. Experimental evidence (9 species/13 studies) shows that digestion strongly influences the proportion and size of otoliths that can be recovered in feces. Number correction factors (NCF) and digestion coefficients have been experimentally determined to reduce the biases in fecal analysis. Correction factors and coefficients have not been determined for diet estimated from stomach contents. QFASA estimates which prey species and amounts must have been eaten to account for the fatty acid composition of the predator. Experimental studies on mammals and seabirds (9 species/10 studies) indicate that accurate estimates of diet can be determined using QFASA. Stable isotope mixing models provide rather coarse taxonomic resolution of diet composition. Prey DNA analysis shows promise as a method to estimate the species composition of diet, but further development and testing is needed to validate its use. Obtaining a representative sample from marine mammal populations is a significant challenge. Therefore, the use of complementary methods is recommended to obtain the most informative results.

Key words: otoliths, feces, fatty acids, stable isotopes, DNA.

Estimating the diets of marine mammals is needed to understand how individuals and populations respond to ecological and environmental variability and their functional roles in marine ecosystems (Bowen 1997). An understanding of diet is also of practical importance in marine mammal conservation (Parrish *et al.* 2002) and to evaluate the contribution of marine mammal predation to sources of natural mortality in prey populations, some of which may be commercially fished or of conservation concern (*e.g.*, Trzcinski *et al.* 2006). In many terrestrial and avian predators, the species composition of diets can be estimated from direct observation of feeding or provisioning, although indirect methods are also used (Pierce and Boyle 1991). Although there are a few exceptions (*e.g.*,

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sea otters, *Enbydra lutris*; Estes *et al.* 1982), diet estimation in free-ranging marine mammals relies almost entirely on indirect methods because there are limited opportunities to directly observe what they eat (Pierce and Boyle 1991, Bowen and Siniff 1999). The oldest and most common methods, and still widely used, involve the recovery of prey hard parts (*i.e.*, calcified structures) from stomach contents, intestines, and feces that are resistant to digestion (Scott 1903, Fitch and Brownell 1968). Less commonly, prey hard parts recovered from spewings (regurgitates) are also used (*e.g.*, Gales and Pemberton 1994, Longenecker 2010). More recently, several chemical methods have been developed. These include the analysis of stable isotopes of carbon and nitrogen (Hobson *et al.* 1997), quantitative fatty acid signature analysis (QFASA, Iverson *et al.* 2004), and the analysis of prey DNA recovered from stomachs and feces (Deagle and Tollit 2007). Finally, animal-borne video has been used to obtain estimates of the diet of several pinnipeds (Bowen *et al.* 2002, Madden *et al.* 2008). Although small sample sizes (both numbers of animals and the sampling rate of video) currently limit its use, video can help to validate estimates made using other methods and may provide reliable independent estimates of diet as battery technology improves. To date, none of the animal-borne estimates of diet have been experimentally validated.

All diet estimation methods make assumptions, have requirements that must be met to generate the best estimates, and have both advantages and disadvantages (Table 1). With the exception of the use of DNA (a rapidly developing method), these methods have been extensively reviewed (*e.g.*, Pierce and Boyle 1991, Pierce *et al.* 1993, Hobson *et al.* 1994, Bowen and Siniff 1999, Bowen 2000, Santos *et al.* 2001, Budge *et al.* 2006, Iverson 2009, Tollit *et al.* 2010). Although the methods differ in many ways, all of the indirect methods currently in use are subject to bias arising from both features of the methods and our ability to sample the diet representatively from wild populations. Given the requirements and assumptions associated with approaches to estimating diet (briefly summarized in Table 1), it is important to understand the accuracy of these methods and the circumstances in which they may be unreliable.

Although the methods used to estimate marine mammal diets have been extensively reviewed, the emphasis has been on application of the methods. What has not been done is to evaluate the experimental evidence on how well the methods work and where they are less successful, although Tollit *et al.* (2010) made reference to this aspect.

Our objectives in this paper are two-fold. First, we critically review feeding experiments with marine mammals that have been conducted to validate methods used to estimate the species composition of diets. Although *in vitro* studies of hard part digestion have been informative (*e.g.*, Jobling and Breiby 1986), those studies have largely been superceded by *in vivo* studies using marine mammals. To date, most of the experimental work has been done on the methods that use recovered hard parts and fatty acids to estimate diet. QFASA is a relatively new method such that fewer experiments have been conducted to date. Therefore, we include in our review, experiments that have been conducted on another mammal and several species of seabirds to broaden the generality of our conclusions. The use of stable isotope mixing models has not been validated in marine mammals. Therefore, we include several studies to indicate their performance in other taxa. Second, we review sources of uncertainty and bias in diet estimation and what can be done to reduce these. We focus on methods using

Table 1. Strengths and limitations of methods used to estimate the diets of marine mammals (after Tollit *et al.* 2010).

Method	Dietary history	Species composition	Prey size	Requirements	Strengths	Limitations
Feces, prey hard parts	Last few meals	yes	yes	<ul style="list-style-type: none"> <li>reference collection of prey species otoliths and bones</li> <li>otolith size measurements</li> <li>otoliths-prey size regressions</li> </ul>	<ul style="list-style-type: none"> <li>large sample size possible</li> <li>nonlethal collection relatively inexpensive to collect and process</li> </ul>	<ul style="list-style-type: none"> <li>prey must have species-specific hard parts and these must be ingested</li> <li>hard parts must resist digestion</li> <li>correction factors to reduce bias caused by partial erosion and complete digestion must be estimated</li> <li>correction factors not available for all prey species</li> <li>false positives and negatives possible</li> <li>may not be representative of species with long foraging trips</li> <li>demographic traits of individuals unknown</li> </ul>

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Table 1. (Continued)

Method	Dietary history	Species composition	Prey size	Requirements	Strengths	Limitations
Stomachs, prey hard parts	Last few meals	yes	yes	<ul style="list-style-type: none"> <li>• reference collection of prey species otoliths and bones</li> <li>• otolith size measurements</li> <li>• otoliths-prey size regressions</li> </ul>	<ul style="list-style-type: none"> <li>• moderate-large sample sizes possible</li> <li>• demographic traits of individuals known</li> <li>• relatively expensive to collect samples</li> </ul>	<ul style="list-style-type: none"> <li>• animals must be killed</li> <li>• prey must have species-specific hard parts and these must be ingested</li> <li>• hard parts must resist digestion</li> <li>• correction factors to reduce bias, but these are not usually available</li> <li>• false positives and negatives possible</li> <li>• may not be representative of species with long foraging trips</li> <li>• often many empty stomachs</li> <li>• differential digestion may further bias results</li> </ul>

(Continued)

Table 1. (Continued)

Method	Dietary history	Species composition	Prey size	Requirements	Strengths	Limitations
Stable isotopes	Days to years depending on tissue	generally no, but exceptions for simple diets	no	<ul style="list-style-type: none"> <li>fractionation factors for tissues</li> <li>reference isotope levels from lower trophic levels</li> </ul>	<ul style="list-style-type: none"> <li>integrates diet over time</li> <li>used as independent check of trophic level</li> </ul>	<ul style="list-style-type: none"> <li>trophic levels are relative to carbon source which must be measured</li> <li>false positives and negatives possible</li> <li>composition and size of prey not known</li> </ul>
Fatty acids	Days to months, depending on species and life history	yes	some coarse resolution possible	<ul style="list-style-type: none"> <li>distinguishable prey fatty acid signatures</li> <li>calibration coefficients (CC) to account for predator metabolism</li> <li>prey fat content</li> <li>predator adipose tissue</li> </ul>	<ul style="list-style-type: none"> <li>integrates diet over weeks-months</li> <li>sampling location less likely to bias composition</li> <li>demographic traits of individuals known</li> </ul>	<ul style="list-style-type: none"> <li>detection level of rare prey still being evaluated</li> <li>false positives and negatives possible because of long integration time, location of foraging less well defined</li> <li>only coarse resolution of prey size</li> <li>estimates sensitive to CC and fatty acid set</li> </ul>

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Table 1. (Continued)

Method	Dietary history	Species composition	Prey size	Requirements	Strengths	Limitations
DNA	Last few meals	no	no	<ul style="list-style-type: none"> <li>• reference species-specific genetic primers</li> <li>• un-degraded prey DNA</li> </ul>	<ul style="list-style-type: none"> <li>• species identification with high accuracy possible</li> <li>• demographic traits can be determined</li> </ul>	<ul style="list-style-type: none"> <li>• can be difficult to isolate suitable DNA from stomach or feces</li> <li>• presence only at this point, but quantitative methods are being developed</li> <li>• false positives and negatives possible</li> <li>• size of prey not known</li> </ul>

recovered prey hard parts and fatty acids because these methods are currently the only ones that provide quantitative estimates of the species composition of prey consumed. Such estimates are usually expressed as percentage by wet mass of prey consumed, which can easily be converted to an estimate of the energy consumed if required. Other measures of diet, such as frequency of occurrence of prey, generally fail to provide reliable estimates of the proportion by mass of prey consumed (Tollit *et al.* 2007). Although the experimental evidence comes mainly from pinnipeds, we expect the results will apply to odontocetes, other marine mammal taxa, and other fish eating predators.

#### DIET COMPOSITION FROM RECOVERED HARD PARTS

The value of prey hard parts recovered from gastrointestinal tracts and feces to infer the diet of marine mammals, seabirds, and terrestrial carnivores has been appreciated for decades. When fish and cephalopods are consumed, sagittal otoliths and beaks, respectively, tend to be the most useful prey structures. This is because these structures permit both species identification and size to be determined. More recently, an "all structures" approach has been used to gain a better understanding of the species eaten, given that the otoliths of some prey species are completely digested leading to false negatives or a biased view of their importance (Pierce and Boyle 1991, Scharf *et al.* 1998, Browne *et al.* 2002, Tarkan *et al.* 2007).

Despite their value, prey hard parts are modified during the process of digestion. This is because the mammalian stomach is a strongly acidic environment, pH ~2–4, designed to reduce food to the point where it can be absorbed by the intestines (Robbins 1983). Although enzymatic digestion occurs primarily in the small intestine, those enzymes function optimally at a neutral pH of 6–8. Therefore, the effects of acid digestion on calcified structures, such as otoliths, are limited to the stomach (Robbins 1983).

*In vitro* studies and experiments using marine mammals show that otoliths and other calcified prey structures are either completely digested or are partly eroded during passage through the stomach (*e.g.*, Prime 1979, da Silva and Neilsen 1985, Jobling and Breiby 1986, Jobling 1987). Over the past several decades a number of *in vivo* experiments have been conducted to evaluate the implications of the effects of digestion on the estimates of diet.

#### *Stomach Contents*

Stomach-content analysis has the longest history; however, because it is necessary to sacrifice animals to fully examine stomach contents, only two experiments have been done on sources of variation and the nature of biases that may influence reliable estimation of diet. Bigg and Fawcett (1985) fed squid (*Loligo opalescens*), herring (*Clupea harengus pallasii*), and sockeye salmon (*Oncorhynchus nerka*) to five northern fur seals (*Callorhinus ursinus*) and found that digestion and passage rates of squid beaks and otoliths from the stomach differed significantly, but were also highly variable among individuals. Salmon otoliths and other hard tissues were either completely digested or passed after 9 h whereas, squid beaks were retained for more than a day. Differential retention of squid beaks, also indicated from stomach contents of wild caught seals (*e.g.*, Pitcher 1981), would

clearly bias estimates of the species composition in the diet. Murie and Lavigne (1986) conducted experiments on 13 individuals of three pinniped species, mainly gray seals (*Halichoerus grypus*), to examine how otoliths recovered in the stomach could be used to infer food consumption and diet. They found that within 3 h of feeding about 99% of herring otoliths fed were recovered in the stomach, however, beyond 3 h the number of herring otoliths recovered in the stomach declined linearly as a function of time and none were found after about 12 h. Intact feces did not contain the missing otoliths indicating that they had been completely digested in the stomach. These experiments also revealed that the number of skull-recovered otoliths (*i.e.*, non-eroded otoliths) declined linearly and by 6–9 h post feeding, all otoliths would have been exposed to digestion. They concluded that correction for complete digestion would be needed to accurately reconstruct the composition of food eaten. Although several decades have passed since this work was published, further experiments have not been conducted to estimate correction factors that would permit unbiased estimates of diet from stomach contents. We do not encourage feeding experiments that require the death of individuals; however other approaches to such experiments might be possible and could allow correction for the very significant effects of digestion.

Erosion of hard parts in the stomach will introduce bias in estimates of the composition of diet, but the extent and direction of that bias will depend, among other things, on the types (*i.e.*, robustness of otoliths or other hard parts ingested) and amounts of prey eaten. Differential rate of passage of otoliths of different species from the stomach will result in bias because once some hard parts have left the stomach, the remaining contents will not accurately reflect consumption. This bias is less likely to occur in fecal analysis, as all otoliths that survive digestion will ultimately be represented in feces, providing enough are collected (Prime and Hammond 1987).

Although hard-part recovery from stomachs is the most common approach, there are exceptions to this. For instance, the diet of the walrus (*Odobenus rosmarus*) has been assessed using soft tissues in stomach contents, identified from anemones, worms, clams, snails, sea cucumbers, and tunicates. Sheffield *et al.* (2001) used *in vitro* digestion experiments to test the hypothesis that walrus prey remain equally identifiable over time in the stomach and that within prey type, prey remain equally identifiable independently of initial size. They rejected their first hypothesis. All Sipunculid worms and polychetes were not identifiable after only 2 h and 3 h, respectively, whereas all soft tissue of clams remained identifiable after 2 h and 50% were still identifiable after 6 h. Snails and crustaceans remained identifiable throughout the 6 h digestion trial. As predicted, larger prey within species remained identifiable for longer. These results have important implications and suggest that previous studies likely introduced biases with respect to the species composition, size and percentage of prey consumed by walruses. In a recent study, comparing stomachs containing fresh remains to those with digested remains, the effects of differential digestion of prey species on estimates of walrus diets were clearly evident, with fresh stomachs containing twice as many taxa and different proportions as those with digested contents (Sheffield and Grebmeier 2009).

#### *Fecal Contents*

A number of experiments have been done to evaluate the extent to which hard parts, mainly otoliths and cephalopod beaks, recovered from pinniped feces can

be used to estimate diet (Table 2). Work by Prime (1979) was among the first to experimentally evaluate the extent of digestion of otoliths recovered from seal feces. A single harbor seal (*Phoca vitulina*) was fed whole specimens of four species of gadoid fish resulting in a recovery rate of 86% of otoliths fed. From this observation, he inferred that the remainder of otoliths had been completely digested. By comparing otoliths fed to those not fed from the same specimens, he also concluded that recovered otoliths were eroded, resulting in an underestimation of the size of prey eaten. Recognizing the potential effect of otolith robustness on digestion, da Silva and Neilson (1985) fed Atlantic herring (*Clupea harengus*), a species with fragile otoliths, to a harbor seal and found that only 4% of otoliths consumed were recovered in feces. Although this recovery rate was negatively biased partly due to inactivity of seals during the experiment (Harvey 1989, Bowen 2000, Grellier and Hammond 2006), it served to highlight one source of variability in estimating digestion coefficients. Subsequently, Prime and Hammond (1987) conducted a series of feeding trials using a single gray seal fed species which differed in the robustness of their otoliths and estimated the first set of species-specific digestion coefficients to account for partial erosion of otoliths.

Experiments to estimate the effects of digestion on otoliths and cephalopod beaks recovered from feces have been conducted on three phocid and six otariid species (Table 2). Harvey (1989) conducted the first series of trials on a wide range of prey species fed to six harbor seals. He found that otolith recovery rate in feces varied significantly among prey species as a function of otolith robustness, and confirmed differences in species-specific reduction in otolith size. Also using harbor seals, but different prey species, Tollit *et al.* (2007) and Phillips and Harvey (2009) confirmed the sources of bias identified by Harvey, but also found that otolith recovery rates and degree of erosion differed by prey length within species. Recognizing that all otoliths within species are not equally eroded during digestion, Tollit *et al.* (2007) developed grade-specific digestion factors to account for the differing degrees of erosion. They also examined the extent to which experimentally derived factors were representative of those in the wild by comparing the distribution of grades from experimental and wild recovered otoliths. Although 34% of cod and whiting otoliths were graded high (*i.e.*, as having little digestion) in the captive experiments, only 24% and 6% of otoliths recovered in the wild were given this grade, suggesting that experimentally derived coefficients may not be entirely representative.

Experiments in which seals were fed mixed diets (e.g., Prime 1979; Berg *et al.* 2002; Tollit *et al.* 1997, 2007), thought to be more representative of how seals feed in the wild, rather than single species also found that otolith recovery rates and degree of partial erosion differed by prey species. Experiments with fur seals and sea lions provided further evidence of the need to correct for the biases introduced by digestion of otoliths and served to underscore the general nature of such effects (Table 2). In addition to prey species effects, experiments on fur seals and sea lions indicated that there are strong predator species effects on the magnitude of biases caused by otolith digestion (Table 2). Recovery rates of otoliths from several species of otariids were considerably lower than that found in harbor seals (e.g., Gales and Cheal 1992, Casper *et al.* 2006). Digestion also reduces the number of cephalopod beaks that are recovered in scats, but the effects are less than on otoliths

Table 2. Feeding experiments<sup>a</sup> to evaluate the accuracy of diet composition estimates from recovered otoliths and squid beaks from feces.

Species	Number animals	Diet	Results	Source
Phocidae Harbor seal, <i>Phoca vitulina</i>	6 (4–8 yr)	11 species fed separately, substituted for maintenance meal	<ul style="list-style-type: none"> <li>• otolith recovery rates differed by species (24%–89%), function of otolith robustness</li> <li>• squid beak recovery rate 44%</li> <li>• recovery rates differed by pairs of seals fed together</li> <li>• otolith length reduction from 16% to 44%; no beak length reduction</li> <li>• passage time: &gt;90% recovery ≤24 h</li> </ul>	Harvey 1989
Harbor seal	7 (1 adult M, 2 adult F, 1 juvenile M, 3 juvenile F)	9 species fed in mixed pairs over 2–3 d	<ul style="list-style-type: none"> <li>• otolith recovery rates differed by species (7%–91%) and size within species</li> <li>• recovery rate for squid and octopus beaks 70% and 84%, respectively</li> <li>• otolith length reduced 27.5% (range 10%–76.5%) differed by species</li> <li>• otoliths length reduction grade-specific</li> <li>• without correction mean mass of prey underestimated by 48% (–4% to –69%)</li> </ul>	Tollit <i>et al.</i> 1997
Harbor seal	4 (2 adult F, 2 subadult M)	5 species fed separately	<ul style="list-style-type: none"> <li>• otolith recovery rates varied (23%–77%) and differed by species</li> </ul>	Cortrell <i>et al.</i> 1996
Harbor seal	7 (2 subadult M, 5 adult M)	7 species fed separately	<ul style="list-style-type: none"> <li>• otolith recovery rates differed by species (10%–85%)</li> <li>• mean reduction in otoliths length 20% and differed by species (1.4%–35%)</li> <li>• squid beak recovery rate 90%</li> </ul>	Phillips and Harvey 2009

(Continued)

Table 2. (Continued)

Species	Number animals	Diet	Results	Source
Gray seal, <i>Halichoerus grypus</i>	7 (adult F)	18 species fed separately or in pairs on 3 occasions	<ul style="list-style-type: none"> <li>• otolith recovery rates differed by species (31%–100%) and length within species</li> <li>• squid beak recovery rate 94%</li> <li>• otolith digestion coefficients were species- and grade-specific</li> <li>• no significant length reduction of squid beaks</li> <li>• passage rates were highly species specific over the first 40 h</li> </ul>	Grellier and Hammond 2006
Northern elephant seal, <i>Mirounga angustirostris</i>	2 (juvenile), no access to water	8 species fed separately	<ul style="list-style-type: none"> <li>• 12% of otoliths recovered,<sup>b</sup> but 87% of squid beaks</li> <li>• No otoliths recovered of some prey species</li> <li>• No reduction in length of squid beaks, but 40% reduction in length of some fish prey species</li> <li>• Overall only 1.4% otoliths recovered in scats</li> </ul>	Harvey and Antonelis 1994
Otariidae California sea lion, <i>Zalophus californianus</i> / South American fur seal, <i>Arctophoca australis gracilis</i>	2/2 (all adult F), animals had no access to water	2 species fed separately	<ul style="list-style-type: none"> <li>• otolith recovery rates differed by prey species (38%–45%)</li> <li>• otolith length reduction of 12%–17%</li> <li>• using recovered otoliths, fish length underestimated by 16% and mass by 35%</li> </ul>	Dellinger and Trillmich 1988

(Continued)

Table 2. (Continued)

Species	Number animals	Diet	Results	Source
New Zealand fur seal, <i>Arctophoca australis forsteri</i>	3 (2 adult M, 1 adult F)	7 species fed separately	<ul style="list-style-type: none"> <li>• otolith recovery rate<sup>c</sup> varied by species (6%–83%)</li> <li>• otolith length reduction 14%–43%, large variation among animals</li> <li>• squid beak recovery rate 60%, no length reduction</li> <li>• without correction for digestion all species underestimated 20%–35%</li> </ul>	Fea and Harcourt 1997
Australian sea lion, <i>Neophoca cinerea</i>	2 (adult M and F)	6 species fed separately	<ul style="list-style-type: none"> <li>• otolith recovery rates differed by species, but &lt;2% for all species and zero for two species</li> <li>• reduction in otolith lengths varied by species (14%–40%)</li> <li>• squid beak recovery rate highly variable with no length reduction</li> <li>• octopus beak recovery indicated size selective feeding with avoidance of larger prey</li> </ul>	Gales and Cheal 1992
California sea lion, <i>Zalophus californianus</i>	5 (1 adult M, 3 adult F, 1 juvenile F)	11 species fed separately	<ul style="list-style-type: none"> <li>• recovery rates of squid beaks 73%, otoliths 51%</li> <li>• highly variable recovery rates by species (0%–100%)</li> <li>• reduction in otolith lengths (17%–48%), no change in cephalopod beaks</li> <li>• passage rates vary among prey and highly variable among seals</li> </ul>	Orr and Harvey 2001

(Continued)

Table 2. (Continued)

Species	Number animals	Diet	Results	Source
<i>Anctophoca</i> spp.	4 (adult M)	7 species fed in two mixed diets of 3 and 5 species	<ul style="list-style-type: none"> <li>only 64% of scat contained otoliths that could be used of diet estimation</li> <li>otolith recovery rates &lt;9% for all species and some species were not represented in scats</li> <li>27% of otoliths too eroded to identify</li> </ul>	Casper <i>et al.</i> 2006
Steller sea lion, <i>Eumetopias jubatus</i>	2 (juvenile F)	4 fed in pairs	<ul style="list-style-type: none"> <li>otolith recovery differ by species (6%–74%) and higher in active <i>vs.</i> inactive animals</li> </ul>	Tollit <i>et al.</i> 2003
Steller sea lion	4 (2 juvenile F, 2 adult F)	9 fed singly and mixed diets	<ul style="list-style-type: none"> <li>otolith recovery rates differed by species (13%–93%), recovery rate highly variable among animals</li> <li>squid beak recovery rate 96%</li> <li>without correction estimates of diet were unreliable</li> <li>application of NCFs produced estimates that were usually within 20% of that fed</li> </ul>	Tollit <i>et al.</i> 2007

<sup>a</sup>Experiments involving two or more animals.

<sup>b</sup>Recovery of hard parts from vomitus (spewings), lavage and scats.

<sup>c</sup>Recovery of hard parts from spewings and scats.

(Table 2), with correction factors similar to those of fish species with robust otoliths. However, unlike otoliths, there is little evidence for a reduction in size of beaks. Despite species differences in magnitude of biases, taken as a whole, the experimental evidence indicates broad agreement across pinniped species on the nature and direction of biases associated with reconstructing diet from recovered otoliths and cephalopod beaks.

In addition to providing evidence of bias, these experiments also provide data that are used to estimate bias-reducing correction factors. Corrections for complete digestion of otoliths, (number correction factors, NCFs), were reviewed and summarized by Bowen (2000). More recent experiments have added to and refined many of those estimates (Orr and Harvey 2001, Grellier and Hammond 2006, Tollit *et al.* 2007, Phillips and Harvey 2009). Digestion coefficients to account for the partial erosion of otoliths and squid beaks have also been estimated for a large number of prey species (*e.g.*, Harvey 1989, Harvey and Antonelis 1994, Tollit *et al.* 1997, Orr and Harvey 2001, Grellier and Hammond 2006, Phillips and Harvey 2009).

Given the problems associated with otoliths, some authors have attempted to improve detection of prey by analyzing other calcified structures that resist digestion and are diagnostic of the species consumed (Pierce and Boyle 1991). Cottrell *et al.* (1996) found that otoliths accounted for only 17% of identified taxon-specific hard parts recovered from harbor seal feces. Other fish structures (*e.g.*, vertebrae) were useful in estimating the incidence of prey in the diet, however, only a few other structures (*e.g.*, dentary, atlas-axis) provided information on the number of prey consumed, but not in all prey species fed during the experiment. Several other studies have also found that relying on otoliths alone resulted in significantly fewer prey being detected than if other structures (*e.g.*, vertebrae, dentary, and teeth) were also used. Tollit *et al.* (2003, 2007) found that using other structures had the greatest benefit in prey species with fragile otoliths such as salmon (*Oncorhynchus* spp.) and sandlance (*Ammodytes hexapterus*). Similarly, Phillips and Harvey (2009) found that recovery rates of prey fed were greater when based on all diagnostic structures rather than when derived from otoliths only, but that the effect was most pronounced for salmon with a 9.5-fold increase in recovery rate. These results suggest that developing recovery rate corrections based on multiple structures can improve the accuracy of diet estimation and that more effort to develop such factors may be warranted. On the other hand, the multiple structures approach increases analyses time and the cost of establishing reference material for identification and the estimation of prey size from the additional structures.

#### *How Good Are Estimates?*

Experimental studies can also be used to determine the accuracy of estimated diets by comparing estimated diet composition with the known proportions of prey species fed. Dellinger and Trillmich (1988) found that, using otoliths recovered from scats, prey lengths were underestimated by 15% and mass by 35%. Nevertheless, they concluded that the proportion of herring to total prey fed could be accurately estimated. A close inspection of the data in their figure 3, however, calls into question this conclusion. Although the slope of their

regression is near 1.0, the variance about the line is so large as to be uninformative. Gales and Cheal (1992) found that a comparison between the actual diets fed to sea lions to those estimated from recovered otoliths and squid beaks indicated that fish were greatly underrepresented and cephalopods were overrepresented. The species composition of the estimated diet was further biased, as several species that had been fed did not appear in the scats (false negatives). The authors concluded that scat analysis was "clearly a poor method for estimating the diet of the Australian sea lion." Harvey and Antonelis (1994) concluded that scats could not be used to estimate the composition of the diet of northern elephant seals (*Mirounga angustirostris*) as squid beaks accumulated in the stomach and most otoliths were completely digested. Casper *et al.* (2006) found that reconstruction of the diet experimentally fed to fur seals from otoliths and beaks recovered in scats resulted in seriously biased estimates. For example, fish with fragile otoliths were represented as incidental even though they accounted for a significant fraction of the diet. These studies underscore the need to correct for the effects of digestion.

Tollit *et al.* (1997) estimated digestion coefficients for seven species of fish, and further refined those coefficients into three grades depending on the degree of otolith erosion. Using recovered otoliths without correction, estimates of the mass of prey species eaten by harbor seals were underestimated by 4%–69%. However, when graded coefficients were applied, species-specific errors ranged from –11.5% to 18.4%. Although the application of grade-specific digestion coefficients improved the accuracy of estimates, Tollit *et al.* (1997) noted that most prey species showed large individual variation in the degree of erosion of otoliths from similar sized fish. This feature presumably accounted for part of the residual error.

Tollit *et al.* (2007) applied experimentally estimated NCFs to evaluate the accuracy of diet reconstruction from otoliths recovered in scats of Steller sea lions (*Eumatopias jubatus*). Using recovered otoliths without NCFs resulted in large errors (~2-fold differences) in the estimated biomass of prey species consumed, with robust-otolith species being overrepresented and those with fragile otoliths being underrepresented. However, after applying NCFs, 13 of 16 comparisons of the percentage of four prey species in the diet were within 5% of that fed.

Phillips and Harvey (2009) developed a simple simulation model to test the accuracy of diet estimation in harbor seals. Assuming that the scats recovered during their experiments were representative of a series of scats collected from the wild, they subsampled 5–40 scats five times to simulate field collections and then compared the estimated diet to that fed. Using the split-sample, frequency of occurrence method, the mean percent difference of individual prey species was 16% and variability did not decrease with increasing sample size. Using biomass reconstruction, they found that estimates of biomass of prey consumed by seals, corrected for otolith erosion and complete digestion (using NCFs from all bony structures recovered), differed from the true biomass by only 3.4% (range among species not presented).

In summary, experimental data demonstrate that without correcting for the effects of digestion, estimates of diet composition derived from recovered hard parts (even all structures) will contain large errors and be subject to bias (*e.g.*, species with robust otoliths are overestimated while those with fragile otoliths are underestimated or not identified), both of which will have consequences for

the reliability of conclusions. However, once corrected for the effects of digestion, reasonably accurate estimates of overall diet composition are possible.

#### *Factors Affecting Digestion of Hard Parts*

A number of factors can influence estimates of digestion coefficients and NCFs. As indicated above, some pinniped species differ in the degree to which they digest otoliths and other prey parts that are consumed. Based on experimental results, recovery rates in Northern elephant seals, and some fur seals and sea lions are low compared to that found in harbor seals and grey seals (Table 2). To some extent, species differences in recovery rates may reflect differences in the prey species used in these experiments, but Marcus *et al.* (1998) found that recovery rates in grey seals were significantly lower than in harbor seals, even though both species were subject to the same experimental environment and fed the same prey species. Studies have also found significant intraspecific, individual variation in recovery rates and reduction in size of otoliths (Grellier and Hammond 2006, Tollit *et al.* 2007). Such individual variation underscores the need to use larger numbers of animals to gain a better understanding of the true variance associated with digestion coefficients and NCFs.

The method used to feed otoliths to seals can also influence digestion of prey hard parts. Grellier and Hammond (2005) compared an experimental otolith-carrier species to *in situ* otoliths of haddock (*Melanogrammus aeglefinus*), plaice (*Pleuronectes platessa*), and sand eel (*Ammodytes marinus*) otoliths fed to two captive gray seals. They found that carrier otoliths were more digested than those fed *in situ*, with the result that neither fish size nor diet could be accurately predicted. Otolith recovery rates varied among the three prey species, but were not affected by feeding method. Further experiments are needed to confirm this conclusion, given that only two seals were used in this experiment.

Activity level of experimental animals is thought to have accounted for the low recovery rates of otoliths in several experiments. Recovery rates of otoliths from seals held in dry areas during feeding experiments were significantly lower than those provided with the opportunity to swim during the experiment (Harvey 1989, Bowen 2000). However, Tollit *et al.* (2003) found that activity level and the opportunity to swim had no significant effect on the recovery rates of otoliths in two Steller sea lions. Given that only two animals were used, this conclusion should be regarded as tentative. In any case, providing an opportunity for experimental animals to swim during experiments should more closely approach conditions in the wild and has become the norm. Experimental studies on otters (*Lutra lutra*) show that both passage time and recovery rate of atlas vertebrae were significantly affected by activity level (Carss *et al.* 1998).

Meal characteristics, such as frequency, size, composition, and energy density can influence digestion and presumably the recovery rates and degree of digestion of hard parts. Trumble and Castellini (2005) experimentally manipulated feeding level, frequency and diet composition in harbor seals and found that mean retention time of food in the stomach decreased as intake increased for both single species and mixed diets. Thus variation in food intake would alter the duration of exposure to stomach acids and the extent of digestion of otoliths and other hard parts. Meal composition also influenced digestion. Trumble and Castellini (2005) found that as food intake increased, up to 45% more digestible energy was assimilated by harbor seals fed a mixed diet compared to a single-species

Table 3. Feeding experiments to evaluate the accuracy of diet composition estimates from fatty acid signature analysis.

Species	n	Diet	Results	Source
Pinnipeds				
Gray seal	6	<ul style="list-style-type: none"> <li>• 3 species, mixed maintenance diet of herring</li> <li>• CC estimates from seals fed herring</li> </ul>	<ul style="list-style-type: none"> <li>• using gray seal calibration coefficients (CC) predictions of fed diet within 5% of true results did depend on CC set used</li> <li>• accurate estimates dependent on the use of CC</li> </ul>	Iverson <i>et al.</i> 2004
Gray seal	28	<ul style="list-style-type: none"> <li>• mixed wild species, replaced with homogenated test diets by gastric intubation</li> </ul>	<ul style="list-style-type: none"> <li>• using gray seal CC predictions of experimental diet contribution at 10–20 d consistent with that fed</li> </ul>	Cooper 2004
Hawaiian monk seal	10	<ul style="list-style-type: none"> <li>• 3 species, mixed maintenance diet of herring</li> <li>• CC estimates from monk seals fed herring</li> </ul>	<ul style="list-style-type: none"> <li>• fatty acid signatures remarkably altered by diet of Atlantic herring compared to diet of Hawaiian Islands prey</li> <li>• diet switch accurately estimated by QFASA with no false positives</li> </ul>	Iverson <i>et al.</i> 2010
Steller sea lion	7	<ul style="list-style-type: none"> <li>• 5 species, mixed diets</li> <li>• CC derived from 5 individuals fed herring</li> </ul>	<ul style="list-style-type: none"> <li>• best results using SSL derived CC</li> <li>• quality of predictions dependent on FA set used</li> </ul>	Tollit <i>et al.</i> 2006

(Continued)

Table 3. (Continued)

Species	<i>n</i>	Diet	Results	Source
Steller sea lion	3	<ul style="list-style-type: none"> <li>• 7 species in mixed diets</li> <li>• seals lost weight during experiments</li> </ul>	<ul style="list-style-type: none"> <li>• best results using SSL derived CC</li> <li>• Overall prediction good, some false positives and species &lt;5% not reliably estimated</li> <li>• pulse feeding not reliably detected</li> <li>• results difficult to interpret as animals lost mass</li> </ul>	Hoberecht 2006
Harbor seal	21	<ul style="list-style-type: none"> <li>• 2 species, 9 others added for model estimation</li> <li>• no dietary history on seals, fed herring and salmon oil prior to experiment</li> <li>• CC estimated from 4 seals fed herring</li> </ul>	<ul style="list-style-type: none"> <li>• harbor seal CC showed some differences from other phocids</li> <li>• fewest errors (13%) using harbor seal-specific CC and reduced FA sets</li> <li>• using full 11 species prey library, error rate was 12%</li> <li>• reducing size of library reduced error rate</li> <li>• false positives included capelin selected instead of herring, mean error 10%, salmon 15%, and sandlance 5%, but some of these could have reflected diet prior to the experiment</li> <li>• QEASA reliably estimated diet of single substitution, control did not change</li> <li>• Large errors were observed in one of nine estimates</li> </ul>	Nordstrom <i>et al.</i> 2008

(Continued)

Table 3. (Continued)

Species	<i>n</i>	Diet	Results	Source
Other mammals				
Mink	18	<ul style="list-style-type: none"> <li>• 3 artificial diets, mixed</li> </ul>	<ul style="list-style-type: none"> <li>• best prediction without CC for 2 of 3 diets with errors &lt;5%, for third diet prediction underestimated by 30%</li> <li>• with gray CC, errors of 10%–15% for fed diet for all diets (but these are not appropriate for unstructured adipose tissue of mink)</li> <li>• two false positive using gray seal CC at ~5%–8%</li> </ul>	Iverson <i>et al.</i> 2004
Birds				
Murres	26	<ul style="list-style-type: none"> <li>• 2 species, mixed</li> <li>• CC derived from constant diet of silverside fed from birth</li> </ul>	<ul style="list-style-type: none"> <li>• predicted diet within 2% of fed diets</li> <li>• 2% false positive in one diet</li> </ul>	Iverson <i>et al.</i> 2007
Kitiwakes	13	<ul style="list-style-type: none"> <li>• 3 species, mixed</li> <li>• CC derived from murres fed constant diet of silverside from birth</li> </ul>	<ul style="list-style-type: none"> <li>• predicted diet within 5% of fed diets</li> <li>• 5% false positive in one diet</li> </ul>	Iverson <i>et al.</i> 2007

(Continued)

Table 3. (Continued)

Species	<i>n</i>	Diet	Results	Source
Steller's eider	8	<ul style="list-style-type: none"> <li>• 5 species, 2 mixed experimental diets</li> <li>• CC derived from Steller's eiders on constant diet</li> </ul>	<ul style="list-style-type: none"> <li>• CC from both eiders similar but differed somewhat from murres</li> <li>• accurate predictions on initial diet, but some errors associated with change in diet depending on FA subset used and CC</li> </ul>	Wang <i>et al.</i> 2010
Spectacled eider	8	<ul style="list-style-type: none"> <li>• 5 species, 2 mixed experimental diets</li> <li>• CC derived from Spectacled eiders on constant diet</li> </ul>	<ul style="list-style-type: none"> <li>• accurate predictions on initial diet, but some errors associated with change in diet depending on FA subset and CC</li> </ul>	Wang <i>et al.</i> 2010

diet. Lawson *et al.* (1997) found that digestive efficiency was positively correlated with prey energy density in harp seals (*Pagophilus groenlandicus*). Marcus *et al.* (1998) found that otolith recovery rate was greater in both harbor and gray seals fed large meals compared to small ones, but that the degree of erosion did not differ with meal size. Taken together these studies indicate that correction factors will vary among pinniped species, among individuals within species, among prey species, and with foraging tactics used in the wild. Thus, our understanding of the foraging ecology of free-ranging marine mammals should be used to inform the design of future experimental feeding studies (this also applies to fatty acids below) with the goal of producing more appropriate correction factors.

### FATTY ACIDS

The idea that fatty acid composition of predator fat stores might contain information about diet has been around for decades, but a method to use fatty acids to quantitatively estimate the species composition of the diet has only been developed recently. Quantitative fatty acid signature analysis (QFASA, Iverson *et al.* 2004) is essentially a mixture model. It requires information on the fatty acid composition of prey species and of predator fat stores (*e.g.*, blubber in marine mammals), a subset of total fatty acids that reflect dietary sources, calibration coefficients (CC) to account for predator metabolism of ingested fatty acids prior to their deposition in blubber, and a statistical model to minimize the statistical distance between the predator and the weighted mixture of prey species representing the diet. QFASA is based on both biochemical knowledge and assumptions, many of which have been tested through experimental feeding of animals in captivity. The method has also been corroborated with results from other diet estimation methods (*e.g.*, seabird stomachs, Iverson *et al.* 2007; animal-borne video on harbor seals, Iverson *et al.* 2004; stable isotope analyses, Tucker *et al.* 2008) conducted simultaneously, through knowledge of prey fields available (Iverson *et al.* 2006, Thiemann *et al.* 2008), and through computer simulations (Iverson *et al.* 2004, 2006; Thiemann *et al.* 2008). Although the method is relatively new, captive validation experiments of QFASA have now been conducted on four species of pinnipeds (six studies), mink (*Mustela vison*), and on four species of seabirds (two studies, Table 3).

Iverson *et al.* (2004) conducted an experiment with gray seals fed on a maintenance diet of Atlantic herring and then switched to a mixed diet of mackerel (*Scomber scombrus*) and capelin (*Mallotus villosus*). Using CC derived from gray or harp seals fed herring, predictions of the percentages of prey in the diet were within 5% of the true values. However, the results depended on which CC were used (Table 3). Cooper (2004) conducted six experiments on wild-caught juvenile (5–10 mo old) gray seals held captive in pens on Sable Island and fed homogenate test diets for periods of ~20 d. Three preliminary experiments were terminated after a short duration because the fish meal used turned out to contain unnaturally high carbohydrate levels resulting in indigestibility of the diet and thus health issues in the seals. In the other three experiments involving 28 seals, and diets containing only ground fresh fish and fish oil, the final fatty acid composition of the blubber was closer to the experimental diet than the initial blubber sample, although this change was nonsignificant in one experiment. The QFASA model was run on initial and final blubber samples to estimate the

extent to which the model accurately predicted the experimental diet. In all cases, by the end of the experiment, the estimated levels (using QFASA) of the mix of species in wild diets declined to be proportionately replaced by the expected input level of the experimental diet. The experimental diet, although quantitatively represented in both inner and outer blubber layers by 13–20 d, was more strongly represented in the inner half of the blubber layer, indicating more rapid turnover of deposited fatty acids in inner followed by outer layer. Iverson *et al.* (2010) studied 10 captive monk seals, *Monachus schauinslandi*, fed northwest Atlantic herring, of which eight seals were then switched to a diet of California spiny lobster (*Panulirus interruptus*) and Spanish mackerel (*Scomberomorus maculatus*), while two remained as controls on herring. Unfortunately, seals would not eat the spiny lobster offered (although this species was used in QFASA modeling) and only half consumed the mackerel offered. When diets were modeled using QFASA, no false positive identifications occurred in any seal at initial sampling or in the two seals maintained on herring at the final sampling (*i.e.*, herring only feeders). In the seals that consumed the experimental diet, the new diet was accurately predicted (within 2% of that fed). Nordstrom *et al.* (2008) conducted a series of three feeding experiments using 21 harbor seals and two prey species and nine other species that were not fed but used in QFASA diet estimation. Using harbor seal specific CC, a set of 35 dietary fatty acids, and the full prey library of 11 species, diets in eight of the nine trials (three experiments) were well-estimated with error rates as low as 4%. Reducing the number of species used from the prey library further reduced the error rate. False positives included species with similar FA signatures such as capelin that were selected by the model instead of herring and salmon, and sandlance, but some of these errors also could have reflected the wild diet of seals prior to the start of the experiment, which was unknown. However, one of the nine trials (*i.e.*, seals fed herring, then smelt [*Hypomesus pretiosus*], then herring) revealed a large error with herring being over estimated by 20% and smelt underestimated by ~30%, increasing the overall average error rate to 12% among the nine trials and indicating that more tests are needed to better understand why this occurred (Nordstrom *et al.* 2008).

Two independent experiments have been conducted with the Steller sea lion (Table 4). Tollit *et al.* (2006, and unpublished data) conducted captive feeding studies on 12 juvenile female Steller sea lions (SSL) to evaluate QFASA's ability to identify known diets and to provide information on FA turnover time, deposition rates, and the FA calibration coefficients required for QFASA. They fed five prey species to sea lions. For each animal, 4–14 sequential blubber biopsies ( $n = 92$ ) were collected from the flank, following various periods of pure and mixed diets over long-term (>100 d), medium-term (28–100 d), or shorter feeding "pulses" (<28 d). A prey database much larger than that actually fed was used to test the accuracy and resolution of QFASA. Predictions were promising for estimating the overall diet and diet switches, but with some exceptions for short-term pulsed diet additions and certainly with prey species containing similar fatty acid signatures. QFASA estimates were consistently most accurate at between 56 d and 84 d. Accuracy of QFASA estimates varied between diet groupings, with the highest accuracy for long- and medium-term pure (single species) maintenance diets. Predictions for submaintenance and mixed-diet trials were variable, especially short-term pulsed trials that included prey of similar composition. Hoberecht (2006) conducted similar experiments

on three adult Steller sea lions fed seven species of prey in mixed and pulsed diets. Single species pulsed diets were fed for 10 d prior to sampling blubber. Overall, the study found the QFASA predictions were good, but that there were some false positives, and that species <5% of the diet were not reliably detected. Two-thirds of the pulse feedings were detected but in only one of these was the level found to be accurate compared to the amount fed indicating that further experiments are needed to better understand the circumstances that lead to such errors.

Experiments on other species of mammals and birds have provided further support for the accuracy of QFASA (Table 3). When CCs from grey seals were used, QFASA estimates of diet in mink were within 2%–20% of the expected value as would be expected given the difference between adipose tissue (mink) and blubber (seals). When no CCs were used, the fed diet was within 5% of the expected value in two experiments, but was underestimated by 30% in the third experiment. Iverson *et al.* (2007) conducted feeding trials on two species of sea-birds reared from hatching on known diets (Table 3). Murres (*Uria aalge* and *lomvia*) were fed only silversides (*Menidia menidia*) from hatching and then half were switched to smelt while the remainder continued on silversides. Kittiwakes (*Rissa brevirostris* and *tridactyla*) were fed a mixture of herring and silversides then half were switched to silversides and the other half to smelt. Biopsies of adipose tissue taken at the end of the experiment were used to estimate the resulting fatty acid composition of the birds. QFASA estimates of diet were within 2%–5% of expected diets. However, there was a false positive of smelt (~2%) in birds fed only silversides. Wang *et al.* (2010) found that QFASA accurately predicted the initial diet and diet switches in two species of eiders (*Somateria fischeri*, *Polysticta stelleri*), but that there were large errors in one of five prey species when using the CC of another seabird species. However, the direct application of the results from these eider CC to the wild is problematic as the test diets fed, both initially and during the diet switches, were extraordinarily high in carbohydrate (from a commercial duck feed), which would never be encountered in a naturally feeding marine piscivore. The degree to which unnatural carbohydrate diets affect fatty acid metabolism requires further investigation.

#### STABLE ISOTOPES

Stable isotope ratios of carbon and nitrogen ( $^{13}\text{C}/^{12}\text{C}$  and  $^{15}\text{N}/^{14}\text{N}$ , respectively) are commonly used to provide information on the diet of marine mammals and other vertebrates (Kelly 2000, Tollit *et al.* 2010). Although useful in addressing a variety of ecological questions, stable isotopes of carbon and nitrogen typically cannot provide quantitative estimates of the species composition of diets unless only three prey species are consumed. With only two isotopes, having more than three potential foods creates an underdetermined system with multiple potential solutions (Phillips and Gregg 2001). Mixing models have been developed to estimate the proportional contribution of up to three foods in the diet (Ben-David *et al.* 1997, Phillips and Gregg 2001). Ben-David and Schell (2001) tested two mixing models (Euclidean distance and linear distance) with three diets fed to captive adult mink and resulting isotope values determined from mink blood and fat samples. The results showed that the isotope values from blood and the linear model predicted the fed diet better than the

Euclidean distance model, but neither model resulted in accurate predictions of the proportion of foods consumed. For example, the linear and Euclidean models predicted 30%–39% and 20%–26% salmon, respectively, whereas the true value was 50%.

The standard mixing model for the two isotope systems assumes that the elemental concentrations of source proportions are equal for the two elements. This assumption often may not be true, thus Koch and Phillips (2002) developed an alternative mixing model for two isotopes and three sources that incorporates both isotopic composition and elemental concentrations to determine the proportional dietary contributions of C, N, and biomass for each food source. Koch and Phillips tested their model on the mink experiment conducted by Ben-David and Schell (2001). The estimated proportions of biomass contributing to mink fat (49% salmon, 28% lean beef, 23% beef fat) were close to the known fractions in the controlled diets (50%, 25%, 25%). Thus, the problem with the standard mixing model seemed to be primarily the imbalance in C and N concentrations among the food sources.

When the number of potential foods is large with respect to the number of isotopes, ranges of foods consumed from specific sources can be estimated with probabilistic models (Phillips and Gregg 2003). The source-partitioning model (Iso-Source, Phillips and Gregg 2003) statistically constrains the relative proportions of various sources to diets and evaluates all biomass combinations of each source user-defined increments to identify source combinations that sum to the known isotopic signature of the mixture. These combinations are feasible solutions, from which the frequency and range of potential source contributions can be determined. Although the development of mixing models has increased the range of questions that can be addressed, none are well suited to estimating the diet composition of marine mammals given the number of prey species typically consumed by these predators.

#### PREY DNA

One of the well-known limitations of prey hard-part analysis is the difficulty of detecting species without digestion-resistant hard parts (*e.g.*, Pierce and Boyle 1991). The ability to identify prey-specific DNA (*e.g.*, Casper *et al.* 2007) has led to the development of a new method for estimating the diets of marine mammals. Although still at an early stage, in terms of providing quantitative estimates of the composition of diets, results to date are promising. Identification of prey DNA involves PCR amplification using either group-specific primers or species-specific targets (Tollit *et al.* 2010). A significant challenge with PCR-based methods is the dominance of predator DNA in DNA isolated from feces (Deagle *et al.* 2005, Deagle and Tollit 2007). Group-specific PCR appears to be an efficient way to detect prey species while simultaneously excluding predator DNA (Jarman *et al.* 2004, Deagle *et al.* 2005). Real-time PCR has also been shown to successfully amplify prey DNA without amplifying DNA from the predator (Deagle and Tollit 2007).

Although initial work was limited to detection of prey, quantitative real-time PCR (qPCR) measures the amount of DNA in a scat sample and opens the way to developing methods to estimate the proportion of species in the diet. Deagle

Table 4. Common sources of bias with methods based on hard parts and chemical identification of mammal diets.

Source of bias	Details
False positives	<ul style="list-style-type: none"> <li>• some eroded hard parts can be incorrectly assigned to species</li> <li>• fatty acids of some species may not be reliably discriminated</li> <li>• secondary prey ingestion</li> </ul>
False negatives	<ul style="list-style-type: none"> <li>• fraction of hard parts usually cannot be identified</li> <li>• species without hard parts will not be detected</li> <li>• species for which fatty acids are not available cannot be estimated</li> <li>• degraded DNA may fail to identify a species</li> </ul>
Representative sampling	<ul style="list-style-type: none"> <li>• hard parts and DNA generally represent the last few meals and therefore an unknown fraction of the diet might not be sampled at haul-outs in marine mammals with long and wide-ranging foraging trips</li> <li>• sampling fraction is so small that it may be impossible to accurately characterize the diet variability due to ecological and demographic factors</li> </ul>

and Tollit (2007) quantified the relative amounts of mitochondrial DNA in the tissue of three fish species being fed to two captive Steller sea lions, and then estimated, using SYBR\_Green qPCR assays, the amount of DNA recovered from these prey items in the feces of 23 sea lions. The quantity of extracted DNA was estimated. The experimental diet contained 50% Pacific herring, 36% surf smelt, and 14% sockeye salmon by mass. The percent composition of fish DNA within the sea lion feces averaged  $57.5\% \pm 9.3\%$  herring,  $19.3\% \pm 6.6\%$  smelt, and  $23.2\% \pm 12.2\%$  salmon. Correction factors based on differences in tissue DNA density improved the estimates, but did not remove the observed biases. Tollit *et al.* (2009) applied the PCR, denaturing gradient gel electrophoresis (DGGE), and DNA sequencing methods to extend the number of prey species that could be detected so the method could be used in analyzing scats collected from free-ranging Steller sea lions. They used universal primers in a primary PCR to amplify prey DNA from scats, and then a fish-specific semi-nested set of secondary primers to further amplify the minute quantities of prey DNA, while eliminating the amplification of DNA from the host sea lion and other nonfish species. Prey DNA was recovered from 87% of scats, although the recovery rate declined to 52% in scats considered "old" at the time of collection. The use of prey DNA resulted in an increase of 22% in the number of prey species detected

over what would have been detected by hard parts only. They detected DNA from more than 40 species of fish and cephalopod prey in 110 scats, averaging approximately two prey species per scat and ranging up to five. Although further validation is required, this study demonstrates the feasibility of identifying the dozens of prey species that can be expected in the diets of wild marine mammals. Matejusova *et al.* (2008) collected scats of captive grey seals fed exclusively on an Atlantic salmon diet or a mixture of herring and sprat (*Sprattus sprattus*) diet. Scats were mixed and spiked by combining salmon-exclusive and non-salmon-fed seal scats to obtain material containing 1%, 5%, and 25% of Atlantic salmon. Real-time qPCR was used to detect salmonid prey in the experimentally manipulated scats. The salmon assay detected target DNA in all subsamples of experimental scats (100%, 25%, 5%, and 1% positive for Atlantic salmon) and there was a highly significant negative relationship between Ct values (PCR cycling time) from the salmon assay qPCR and the proportion of Atlantic salmon in the experimental spiked scats. Despite this relationship, estimating the quantity of prey in field scats is only relative because no information is available on the rate of DNA degradation for different prey species during digestion, or degradation of prey DNA in scats in the environment prior to collection. Bowles (2009) conducted two feeding experiments with a total of six Steller sea lions to validate real-time qPCR derived estimates of diet composition. In one experiment, animals were fed a diet of 64.3% herring, 14.3% eulachon (*Thaleichthys pacificus*), 14.3% squid, and 7.1% rockfish (*Sebastes alutus*) by weight. After applying correction factors to the amount of mtDNA in each prey species, recovered DNA in fecal samples indicated a diet of 87.7% herring, 9.9% eulachon, 2.0% squid, and 0.44% rockfish. In the second experiment, two sea lions were fed 10 diets of the same four prey species in sequence. In this experiment, the estimated percentages were always within 12% of the expected amount, similar to the accuracy of the technique based on estimates from mixes of known amounts of DNA. Although some bias is evident in these results, these experiments do indicate that further experimentation along with continued development of molecular prey identification techniques seems warranted.

The strengths and limitations of DNA identification of prey consumed by predators are reviewed by King *et al.* (2008). An important advantage of this approach is that more species are detected in scats than in hard-part identification of the same samples (Tollit *et al.* 2009). Nevertheless, feeding studies on captive sea lions have shown that prey can be detected in scats during a 48 h period after defecation (Deagle *et al.* 2005), indicating that the use of prey DNA may be limited to recent feeding. Deagle and Tollit (2007) also note that the technical difficulty and cost of quantifying DNA from multiple potential prey species may restrict its application. However, new sequencing technologies (*e.g.*, Genome Sequencer (GS)-FLX pyrosequencing technology) can provide an unprecedented amount of sequence data at a low cost from individual DNA molecules in complex mixtures without the need for cloning (Deagle *et al.* 2009). Recently, Deagle *et al.* (2010) pyrosequenced mtDNA markers amplified from feces of captive little penguins (*Eudyptula minor*) fed known diets. Pilchards were the primary fish fed and DNA sequences from pilchard were the most common sequences recovered. Sequences of three other fish fed in constant mass proportions (45:35:20) were all detected, but proportions of sequences (60:6:34) were considerably different than mass proportions in the diet. Correction factors based on relative mtDNA density in the fish did not improve diet estimates. The

authors concluded “that DNA sequences recovered in dietary barcoding studies can provide semi-quantitative information on diet composition, but these data should be given wide confidence intervals.”

#### SOURCES OF VARIATION AND POTENTIAL BIASES COMMON TO ALL METHODS

Several sources of variation and biases are common to all methods used to estimate marine mammal diets (Table 4). False positives can occur in hard-part analysis by incorrectly identifying the species. Freshly removed otoliths are easily identified to species in most cases, although small gadoid and flatfishes can be difficult or impossible to identify to species. The situation becomes more difficult with partially eroded otoliths and there is some subjectivity in the identification to species and thus some level of error can be expected. Nevertheless, the magnitude of false positive errors associated with species identification using hard parts has not been studied. In the case of fatty acids, identification is based on chemical standards and column retention times relative to those standards. Quantification is computer based, but also requires visual assessment of the correct separation and identification of a number of fatty acids. Interlab comparisons and verifications are rare and thus combining prey and predator data from different labs should be assessed further. False positives can occur using QFASA when a species not eaten is identified in the diet (see Table 3). This can occur because prey species may have similar fatty acid profiles and because diet estimates are influenced by the CC and the subset of fatty acids used. False positives also can occur in stable isotope ratio analysis and prey DNA analyses, but their occurrence has not been well documented.

False negatives occur in hard-part analysis when otoliths cannot be identified or recovered and therefore some species that are eaten may not be detected. For example, ~4% of otoliths by number recovered from grey seal stomachs were unidentified flatfish and gadids, or unknown fish (Bowen *et al.* 1993). Unidentified prey accounted for >10% of the estimated minimum number of prey eaten by Juan Fernandez fur seals (*Arctophoca philippii*, Acuna and Francis 1995). Also species without hard parts or those whose hard parts are completely digested will not be detected in the diet (*e.g.*, Dellinger and Trillmich 1988, Sheffield *et al.* 2001). When using QFASA, false negatives can occur if a species that is consumed is not included in the prey library or if two species have fatty acid compositions that cannot be reliably distinguished. The former is more difficult to guard against than the latter which can be tested empirically. To date, prey species used to estimate diets of pinnipeds can generally be statistically identified with high accuracy (*e.g.*, Iverson 2009, Budge *et al.* 2002). Nevertheless, there are exceptions, and because of the high dimensionality of the QFASA model and the application of CCs to the fatty acid values, the way in which species are distinguished in the model may differ compared to when using more common multivariate analyses.

Another source of variation common to all methods is sampling error (Hammond and Rothery 1996, Pierce *et al.* 2007). Uncertainty in estimates of diet resulting from sampling error can generally be reduced by increasing sample size, but the inability to obtain a representative sample of the population may not be possible, resulting in bias. In fact, representative sampling may be the most

important source of bias given the wide-ranging and remote nature of marine mammal foraging and that feeding typically occurs at depth, limiting the portion of the diet that can be sampled. These attributes of foraging would not be a source of bias if diet were homogeneous across time and space, but we have ample evidence that this is rarely if ever the case in marine mammals, as sex, age, reproductive status, season and prey distribution and abundance are common sources of variation (Pierce and Boyle 1991, Bowen and Siniff 1999).

The gray seal provides an illustration of the difficulty of obtaining a representative sample of the diet and how this might differ among populations within species. We anticipate that similar difficulties will be common among other marine mammals, seabirds and perhaps sharks. Off eastern Canada, most gray seals use Sable Island as a central place from which to forage on the continental shelf, although other haul-out sites are also used along the coast of Nova Scotia (Breed *et al.* 2006). Austin *et al.* (2006) used stomach temperature telemetry to estimate meal frequency in adults and determined that, on average, seals ate 1.7 meals per day. Gray seal foraging trips last about nine days at sea followed by about one day hauled out on land (Beck *et al.* 2003, Breed *et al.* 2009). Based on movement and diving behavior (Breed *et al.* 2006, Beck *et al.* 2003), gray seals feed throughout the year with the exception of about one month each during the spring molt and winter breeding season. Although both foraging trip duration and diving behavior show strong seasonality (Beck *et al.* 2003, Breed *et al.* 2009), we ignore this detail for the purposes of illustration. Using these data, we calculate that each individual in the population might consume about 470 meals per year. A population of 250,000 gray seals would eat about 117 million meals per year.

Using the recovery of hard parts in scats as the basis for estimating the diet, we assume that a meal can be distributed over four scats and therefore each scat is likely to represent more than one meal (Phillips and Harvey 2009). This assumption would presumably be similar for stomachs. With fatty acids or stable isotope ratios, each sample would represent more meals because the integration of prey fatty acids and isotope into tissues occurs over periods of perhaps several months (Iverson *et al.* 2004, Nordstrom *et al.* 2008). Therefore, each fatty acid sample might represent about 100 meals. Passage rate of a meal from the stomach typically occurs within 48–72 h in gray seals (Grellier and Hammond 2007). Therefore, with foraging trips of nine days, we should only expect to see a small fraction of meals deposited on the beach in the form of scats (maximum of ~25%). Phillips and Harvey (2009) found that only 5% of scats were recovered on land in a captive setting, suggesting that feces may be preferentially passed at sea. Nevertheless, assuming that this is not the case, of the 117 million gray seal meals, only about 25% might be deposited on the beach, or about 29 million. During the course of a year, an ambitious program might collect scats monthly, resulting in about 300–500 samples per year. This represents  $\ll 0.01\%$  of meals eaten, most of which are not available to be sampled from land. This serves to underscore the difficulty researchers may often face in attempting to accurately estimate the true diet of a marine mammal. This difficulty will be greater if the proportion of a prey species in the diet is small. The situation with fatty acids (and potentially stable isotopes) is better, but still the sampling rate is low. The major difference is that we do not, at least in principle, miss a large fraction of the meals eaten because of long foraging trips and rapid passage of digested food, as meals are stored in the blubber over time. Even so, ~250 fatty

acid samples collected in a year represent only about  $100 \times 250/117,000,000$  or 0.02% of meals consumed.

These calculations could be refined considerably to account for correlation in the diet within and among individuals which could reduce the effective number of meals/individuals that need to be sampled to estimate the species composition of the diet. Of course the situation may be better if the population of interest is much smaller and foraging trips are short relative to passage time of meals. This seems to be the case in the UK where simulations indicate that the short foraging trips of gray seals on the west coast of Scotland are unlikely to result in bias in estimates of diet.<sup>2</sup> Nevertheless, refinements in the assumptions are unlikely to change the conclusion that our ability to obtain a representative sample of wide-ranging species with long foraging trips relative to passage time of food from the gut will be difficult. This situation is perhaps most severe when sampling scats or stomachs from land in the case of pinnipeds or seabirds, but our limited ability to follow cetaceans at sea will presumably result in similar bias given that such a large fraction of scats will not be available.

#### DISCUSSION

The impossibility of collecting all fecal material from wild animals and the extent of digestion of otoliths in captive experiments, led Dellinger and Trillmich (1988) to conclude that "reasonable estimates of absolute numbers of fish ingested by free-living seals from scat analysis" was not possible. Gales and Cheal (1992) concluded that "scat analysis is clearly a poor method for estimating the diet of the Australian sea-lion" since several species fed were not detected in scats. In both cases, recent research indicates that these conclusions were too pessimistic, but it is clear that large errors (including false positives and negatives) and bias in diet composition can occur from the analysis of hard parts unless steps are taken to correct estimates. Although accurate quantitative estimates of diet are needed for many research questions, it is worth noting that, in other cases, knowing that the diet has changed may be sufficient. In such cases, differences in the estimated diet can be informative even though they misrepresent the actual diet.

Experimental evidence clearly demonstrates that during digestion otoliths are partially eroded or completely digested in pinnipeds. Squid and octopus beaks are significantly more resistant to digestion, but correction is also needed for some species. Species without hard parts may have species-specific structures that resist digestion, but other soft-bodied species are completely digested. Together these effects can seriously bias estimates of diets. This is consistent with experimental data in other mammals and seabirds (*e.g.*, Johnstone *et al.* 1990, Zijlstra and van Eerden 1995, Carss and Elston 1996, Carss and Parkinson 1996).

Despite this evidence, researchers have been slow to apply factors to reduce these biases. Some of this reluctance can be attributed to the lack of estimates of correction factors for many prey species commonly consumed by seals and other marine mammals. However, researchers should be aware that the situation has improved considerably since the review of NCFs by Bowen (2000), with the

<sup>2</sup>Personal communication from Sophie Smout, Sea Mammal Research Unit, St. Andrews, U.K., 4 October 2011.

experimental work on gray seals by Grellier and Hammond (2007), continued experimental work on harbor seals (*e.g.*, Phillips and Harvey 2009), and new studies on Steller sea lions (*e.g.*, Tollit *et al.* 2007). There are now estimates of NCFs and digestion coefficients for 54 fish species and seven squid and octopus species. Nevertheless, estimates of correction factors still have not been determined for a number of prey species. The highly variable nature of experimentally derived NCFs and digestion coefficients of otoliths within prey species has also been used by researchers as the basis for not correcting for bias. Although both of these are good reasons to be cautious, neither reason justifies ignoring the known direction and magnitude of resulting bias. One approach to the lack of correction factors for some prey species is to use correction factors for species with similar otolith size and robustness and degree of erosion. This is based on experimental evidence showing that the magnitude of corrections for the effects of digestion is reasonably well predicted by these otolith characteristics (*e.g.*, Harvey 1989, Tollit *et al.* 2007). The large variability within prey species is perhaps more difficult to deal with, but even here using means with the measured variance should improve the accuracy of the estimated diet. Available correction factors are found in the cited references. We have not compiled them as researchers will need to decide for themselves which corrections are appropriate for their species.

To date, the greatest progress has been made in accounting for bias resulting from the effects of digestion on otoliths recovered from fecal samples. By contrast there has been little progress with respect to developing correction factors to reduce bias associated with the analysis of stomach contents. Nevertheless, it is clear that hard parts are both lost and eroded in the stomach and that correction for the differential effects of digestion on the otoliths of different species will need to be done. Until we understand better how digestion affects otoliths and other hard parts of species consumed, estimates of diet from stomach contents are likely to be highly biased and misleading.

Experimental evidence on diet estimates from fatty acids indicates that predicted diets are often quite accurate, particularly when species-specific calibration coefficients are used. However, experimental studies also show that occasionally large errors and false positives or negatives can occur. Further experimental evidence is needed to better understand both the resolution of the method and the circumstances which lead to significant errors. Work done to date indicates that estimates of diet are sensitive to the calibration coefficients used. Thus, a better understanding of the sources of variation in calibration coefficients (*e.g.*, individual effects, effects of lipid concentration in the diet) and their effects on diet is needed. Also, further experiments are needed to refine our understanding of the resolution (*e.g.*, can a prey proportion of, say, 2% be reliably estimated) and time-frames of diet estimates from fatty acids.

Stable isotope mixing models will continue to play a limited role in estimating the proportional contribution of species to the diet of marine mammals, because with only two or perhaps three isotopes the number of food types that can be estimated is small. Nevertheless, stable isotope analysis can play an important role in helping to validate independent estimates of diet derived by other methods. The few experiments that have tested the accuracy of quantitative prey DNA analysis suggest that we can expect to add this technique to the list of those providing estimates of diet composition. However, more research is needed on the effects of DNA deterioration during digestion and in the environment prior to collection on quantitative estimates of prey species.

Based on experimental evidence to date, it seems clear that both the analysis of hard parts recovered from feces and the chemical and statistical analysis of fatty acids of predator and prey contain useful information about the diets of pinnipeds. However, both approaches, as well as the emerging area of prey DNA, are dependent on assumptions that can or have not been adequately tested and can lead to error and bias in estimates of diet. Although we should attempt to improve all approaches, through further experimental studies, the daunting task of representative sampling would suggest that it is a mistake to spend too much time debating which methods are reliable and which are not. Rather, we suggest that the most reliable view of the diet, particularly for wide-ranging species with long foraging trips, will come from the simultaneous use of multiple methods. For example, Tucker *et al.* (2008) compared independent estimates of trophic level of feeding from stable isotopes of carbon and nitrogen with those from QFASA within the same individual as a means of indirectly validating diet composition estimates.

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