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Molecular scatology as a tool to study diet: analysis of prey DNA in scats from captive Steller sea lions

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Abstract

The DNA of prey present in animal scats may provide a valuable source of information for dietary studies. We conducted a captive feeding trial to test whether prey DNA could be reliably detected in scat samples from Steller sea lions (*Eumetopias jubatus*). Two sea lions were fed a diet of fish (five species) and squid (one species), and DNA was extracted from the soft component of collected scats. Most of the DNA obtained came from the predator, but prey DNA could be amplified using prey-specific primers. The four prey species fed in consistent daily proportions throughout the trial were detected in more than 90% of the scat DNA extractions. Squid and sockeye salmon, which were fed as a relatively small percentage of the daily diet, were detected as reliably as the more abundant diet items. Prey detection was erratic in scats collected when the daily diet was fed in two meals that differed in prey composition, suggesting that prey DNA is passed in meal specific pulses. Prey items that were removed from the diet following one day of feeding were only detected in scats collected within 48 h of ingestion. Proportions of fish DNA present in eight scat samples (evaluated through the screening of clone libraries) were roughly proportional to the mass of prey items consumed, raising the possibility that DNA quantification methods could provide semi-quantitative diet composition data. This study should be of broad interest to researchers studying diet since it highlights an approach that can accurately identify prey species and is not dependent on prey hard parts surviving digestion.

Keywords: denaturing gradient gel electrophoresis, *Eumetopias jubatus*, faeces, hard parts, noninvasive, species identification

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Introduction

Determining trophic relationships within an ecosystem is a key part of many ecological studies (Trites 2003); however, obtaining reliable data on diet composition for most species is fraught with difficulties. Pinnipeds are one group of vertebrates whose diet has been extensively studied due to population declines of some pinniped species (e.g. Merrick *et al*. 1997; Sinclair & Zeppelin 2002), declines in numbers of some of their prey (e.g. Orr *et al*. 2004), and because of the overlap between their prey and species targeted by commercial fisheries (e.g. Harwood & Croxall 1988). At present, pinniped diet is primarily studied by morphological identification of prey hard part remains found in scats (cephalopod beaks, fish otoliths and bones) (Olesiuk 1993; Tollit & Thompson 1996; Sinclair & Zeppelin 2002). There are several well-documented problems with data from these studies chiefly resulting from the fact that prey species with robust hard parts, which can readily survive digestion, are likely to be over-represented in scat whereas prey species with less robust or no hard parts are likely to be under-represented or not represented at all (Harvey 1989; Tollit *et al*. 1997). While numerical correction factors can reduce these biases, it has been demonstrated that digestion rates are affected by many different factors (Bowen 2000; Tollit *et al*. 2003), making their application problematic. Alternative methodologies such as stable isotope and fatty acid signature analyses (Hobson *et al*. 1997; Iverson *et al*. 2004) provide less specific, longer term data that are useful in many situations. However, they typically require animal capture and they do not provide the taxonomic

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and/or the numerical resolution that is often obtainable from scat analysis.

Traditional mammalian diet studies (using morphological identification of prey remains in scats) have been carried out in combination with genetic analysis of the predator's DNA present in scats to identify which species of predator the scat originated from (Farrell *et al*. 2000) and to ascertain the sex of the defecator (Reed *et al*. 1997). The use of DNA-based techniques to study diet directly has been carried out mainly in terrestrial invertebrates, where predators are sacrificed and the prey present in their stomachs is identified using polymerase chain reaction (PCR) detection methods (Symondson 2002; Agustí *et al*. 2003; Kasper *et al*. 2004). This destructive approach is clearly less acceptable in mammalian studies and has led to the development of noninvasive methods to detect prey DNA in the scats of vertebrate predators (Höss *et al*. 1992; Jarman *et al*. 2002; Purcell *et al*. 2004). Multicopy nuclear ribosomal and mitochondrial DNA (mtDNA) prey markers have been obtained from whale and penguin scats (Jarman *et al*. 2002, 2004), and single-copy nuclear DNA prey genes (Y chromosome fragments from male white-tailed deer) have been detected in the soft component of female bear scats (Murphy *et al*. 2003). Purcell *et al*. (2004) used DNA extracted from bones found in Pacific harbour seal scats to obtain species-level identification of salmonids. This approach allowed greater resolution than morphological analysis of the salmon bones (which provided identification only to family level), but identification was still contingent on prey hard parts surviving digestion. Detecting the presence of prey DNA in the soft component of scats may provide an alternative means of determining diet that is less affected by biases associated with differential digestion and passage (see Tollit *et al*. 2003) and could also allow for the detection of soft-bodied prey items.

We conducted a feeding trial with captive Steller sea lions (*Eumetopias jubatus*) to further investigate the capacity of genetic techniques to recover prey DNA from scats. Our approach was to look at the reliability of PCR amplification of prey DNA from the scats of animals fed a consistent daily diet made up of several prey species. We analysed several subsamples of each scat to determine distribution of prey DNA in the scats and included novel 'pulse' prey items to monitor the persistence of the genetic signal. Finally, we assessed whether some quantitative estimate of diet composition could be obtained by quantifying the amount of DNA present in the scat through the screening of PCR clone libraries.

Materials and methods

Feeding trial and sample collection

Two female Steller sea lions participated in the trial from July to September 2003 at the Vancouver Aquarium Marine Science Centre. They were housed individually either in a continuously flowing 20 000 L saltwater swim tank with a 2×2 m haul-out platform or in a 1.8×2.5 m grated dry run. The first animal (#F97HA, mean mass 146 kg, 6 years old) was in the feeding trial for 48 days and the second animal (#F00NU, mean mass 131 kg, 3 years old) for 24 days. Six species of prey were used in the trial: Pacific herring (*Clupea pallasii*), surf smelt (*Hypomesus pretiosus*), sockeye salmon (*Oncorhynchus nerka*), walleye pollock (*Theragra chalcogramma*), capelin (*Mallotus villosus*) and Californian market squid (*Loligo opalescence*). The basic daily diet (7–8 kg per day, *c*. 5.5% of body mass) was fed in two meals (at *c*. 9:30 and 14:30) and consisted of herring (47% by mass), smelt (34%), salmon (13%) and squid (6%). This diet was initiated at least 4 days before the first scats were collected. Over most of the trial the diet was fed in two meals which were equal in mass and had the same proportions as the total daily diet. However, during the first 14 days of the trial, Animal 1 was fed the basic daily diet in unequal meals, with only smelt being fed in both meals (meal 1 was 6.25 kg consisting of 60% herring, 32% smelt and 8% squid; meal 2 was 1.75 kg consisting of 57% salmon and 43% smelt). This regime allowed us to evaluate the extent of mixing of prey DNA from different meals. The other variation in the basic diet was the inclusion of novel prey species in place of herring. This was done on three occasions — 1 day where pollock was fed to Animal 1 in place of herring and another 2 days where capelin was fed to Animal 2 instead of herring. The purpose of these novel prey pulses was to determine how long prey DNA would be detectable in scats after consumption (see Fig. 1 for overview of feeding regimes).

During the feeding trial, samples were collected from either individual scats obtained on the dry run/haul-out ($n = 13$), or from scats obtained through swim tank draining ($n = 27$). Four samples were taken during each collection. Three small subsamples (2–3 mL faecal material) were obtained from distinct intact faecal lobes. The remainder of the scat (or random portions of several lobes when volume of scat was prohibitively large, > 250 mL) was mixed to form a final blended sample. All scat samples were preserved in a volume of 95% ethanol three to five times greater than the sample volume.

Several additional scat samples were analysed during the study. Four scat subsamples were collected as control samples from captive sea lions not directly involved in the trial — three from an animal being fed a diet of solely Pacific herring and one from an animal being fed solely pollock. In order to investigate the degradation of DNA in unpreserved scats, components of two large scat samples were left at ambient temperature (high 26 °C, low 13 °C, mean 19 °C) in an open container exposed to sunlight and subsamples ($n = 18$) were preserved in ethanol at times ranging from 0 h to 18 days. Finally, 12 subsamples were taken from various scats collected during the study period (from

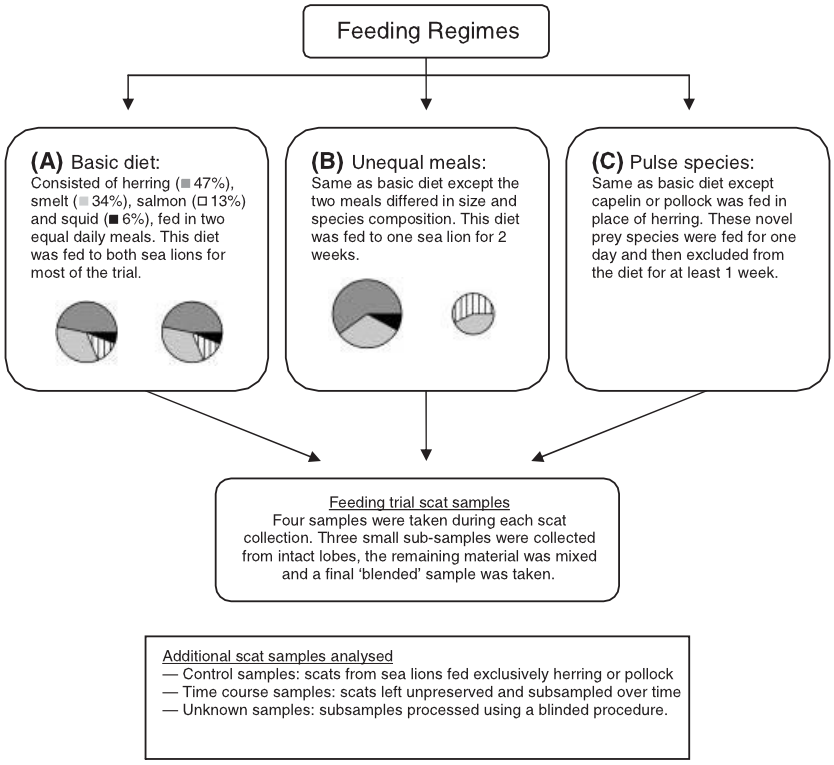

Fig. 1 Overview of feeding regimes and scat samples collected during the current study.

sea lions within and outside of the study that had known diets). These samples were tested using a blinded procedure (i.e. information on prey DNA expected to be in the samples was withheld from the researcher carrying out the laboratory analysis).

DNA extraction, PCR and sequencing

Extraction of DNA from scats was carried out using the QIAamp DNA Stool Mini Kit (QIAGEN). Samples were resuspended in the storage ethanol and then 1.5 mL of the ethanol/scat slurry was removed and centrifuged for 30 s at 4000 × *g*. The ethanol was poured off and the dry weight of the pellet was determined. All remaining steps followed the manufacturer's instructions, except that only half the recommended volume of buffers/InhibitEX tablets was used. The buffer volumes were cut down to reduce the risk of crossover contamination by minimizing the number of pipetting steps and by reducing the volume of liquid loaded into spin columns and tubes. The DNA was eluted in 100 μL Tris buffer (10 mM). In total, DNA was extracted from 194 samples (120 distinct subsamples and 40 blended samples from the 40 feeding trial scat collections, 3 herring-only scats, 1 pollock-only scat, 18 exposure time course subsamples and 12 blind subsamples). Extraction blanks (containing no scat) were included (n = 8) to check for crossover contamination. Scat DNA extractions were performed in a laboratory that had not previously been used for DNA analysis and were carried out before any prey DNA was extracted from tissue. Extraction of DNA from prey tissue was carried out using the DNeasy tissue kit (QIAGEN).

The 3′ end of the mitochondrial 16S ribosomal RNA gene was chosen as a PCR target since we have previously designed conserved primers which will amplify a short DNA fragment from the prey species used in the feeding trial (16S1F + 16S2R; Table 1) and we have also developed a DGGE-based species identification method for this region (B. Deagle *et al*., 2005). In addition to these 'universal' primers, we amplified prey DNA using two sets of group-specific primers (Fig. 2). One of these primer pairs specifically amplifies DNA from the fish prey and the other amplifies only squid DNA. The fish-specific forward primer was designed by aligning the Steller sea lion 16S mtDNA sequence (GenBank Accession no. NC004030) with homologous sequences from the fish prey species fed in the feeding trial (GenBank Accession nos AY799999–AY800003). The resulting primer (16fishF, Table 1) was used in conjunction with 16SR (*c*. 250 bp product). It is completely conserved in the feeding trial fish species but only one out of five base pairs at the 3′ end of the primer match the Steller sea lion and the primer is not conserved in squid. The squid PCR primers we used (Table 1) amplify a region of nuclear 28S ribosomal DNA (*c*. 180 bp product) from squid, but not from other molluscs or more distantly related animal taxa (K. Goldsworthy & S. Jarman, unpublished).

Table 1 PCR primers used in the present study

Pimer name*	Sequence 5′–3′	Target (gene: species)
16S1F	GGACGAGAAGACCCT	mtDNA 16S: sea lion, fish, squid
16SfishF	AGACCCTATGGAGCTTTAGAC	mtDNA 16S: fish in present study
16S2R	CGCTGTTATCCCTATGGTAACT	mtDNA 16S: sea lion, fish, squid
16S2R Clamp	GGGCGGGGGCGGCGGGACGGGCGCGGGG CGCGGCGGGCGCGCTGTTATCCCTATGGTAACT	mtDNA 16S: sea lion, fish, squid
Squid28SF	CGCCGAATCCCGTCGCMAGTAAAMGGCTTC	nuclear 28S rDNA: squid
Squid28SR	CCAAGCAACCCGACTCTCGGATCGAA	nuclear 28S rDNA: squid

*F and R denotes forward and reverse.

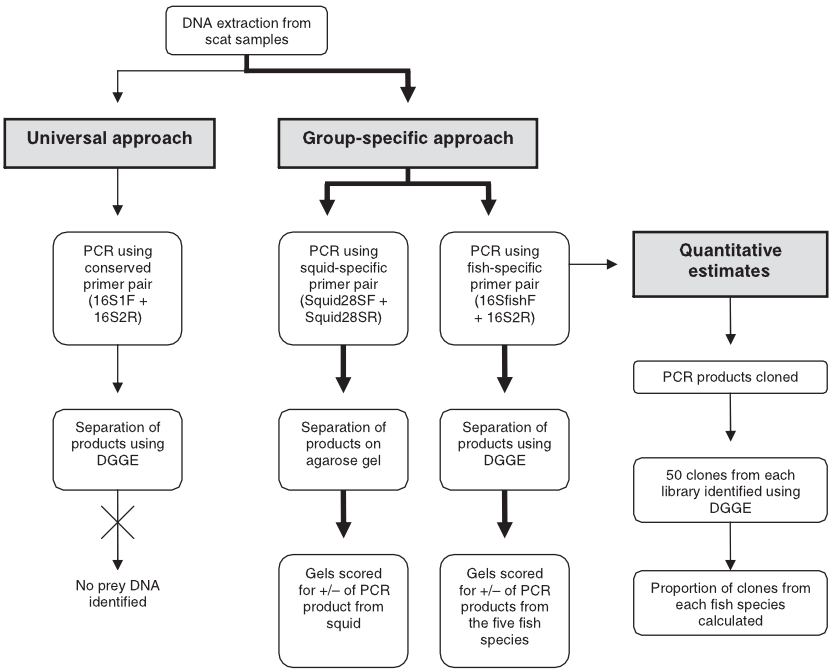

Fig. 2 Overview of genetic analysis performed during the current study. Thick arrows represent analysis carried out on all scat samples, thin arrows show analysis carried out on a subset of samples.

Standard PCRs were performed on 1 μL of DNA extracted from scat in a 25 μL volume containing 0.4 μM of each primer, 0.2 mM dNTPs, 2.0 mM $MgCl_2$, 1 × BSA (New England Biolabs),1 × Ampli*Taq* Gold buffer and 0.625 unit Ampli*Taq* Gold (Applied Biosystems). Thermal cycling conditions were as follows: 94 °C for 10 min then 35 cycles (94 °C, 30 s/55 °C, 30 s/72 °C, 45 s) followed by 72 °C for 2 min. Aerosol-resistant pipette tips were used with all PCR solutions and negative control reactions (extraction controls and a PCR blank) were performed with each batch of PCR amplifications. Samples were separated on a 1.8% agarose gel or gradient acrylamide gels. Sequencing was carried out using the CEQ Dye Terminator Cycle Sequencing Quick Start Kit, employing half reactions; products were electrophoresed on a Beckman Coulter CEQ 2000 automated sequencer. Sequences from the mitochondrial 16S ribosomal RNA gene of the fish prey species were obtained through direct sequencing of PCR products amplified using the primers 16Sar-5′ and 16Sa-3′ (Palumbi 1996).

Denaturing gradient gel electrophoresis

We used denaturing gradient gel electrophoresis (DGGE) to separate unique PCR products in amplifications expected to contain DNA from multiple species (see Myers *et al*. 1987; Lessa & Applebalm 1993). DGGE was performed using the DCode system (Bio-Rad). Acrylamide gels were prepared according to the manufacturer's instructions and poured using a Model 475 Gradient Delivery System (Bio-Rad).

For samples separated by DGGE, the 16S2R primer was redesigned to incorporate a GC clamp (Sheffield *et al*. 1989). Nested PCR was carried out to improve the intensity of the bands obtained from the fish-specific PCR (using the clamped reverse primer) and scat DNA template. Primary

enrichment PCR was conducted using the unclamped primer pairs (16S1F and 16S2R). Cycling conditions were 94 °C for 10 min, then 20 cycles (94 °C/30 s, 56 °C/30 s and 72 °C/1 min) followed by 72 °C for 2 min. The secondary PCR was carried out as for our standard PCRs (see previous section) with the clamped reverse primer and 1 μL of the primary reaction as template. Electrophoretic conditions (percentage acrylamide, gradient range, voltage and length of run) that resulted in clear band separation were determined by experimenting with products amplified from genomic DNA of the prey species (see Results).

Quantitative estimates

Clone libraries were constructed from fish PCR products to quantify the proportions of fish DNA present in eight samples (Fig. 2). The eight samples analysed included five DNA samples from scats collected when the sea lions were fed the basic diet in equal meals and three DNA samples from scats collected when the daily diet was fed in unequal meals. The clone libraries represent template DNA from single scat samples ($n = 5$, three from equal diet feeding regime and two from unequal diet feeding regime) or mixtures of DNA from seven scats ($n = 3$, two from equal diet feeding regime and one from unequal diet feeding regime). The DNA mixtures were included to determine if pooling of DNA from several extractions would provide an average view of diet rather than the snapshot that might be expected from an individual scat. Scats that potentially contain pollock or capelin DNA were not included in this analysis. Standard PCR (as previously discussed) was carried out using 50 μL reaction volumes, 2 μL of scat DNA template and the 16SfishF and 16SR primer pair. PCR products were cloned into the pCR 2.1 TOPO TA cloning vector and transformed into TOP10 chemically competent *Escherichia coli* (Invitrogen). The bacteria were plated and positive transformants recognized using blue/white colour selection. For each sample, 50 white colonies were picked using a pipette tip and suspended in a 20 μL PCR mixture containing the primers (16SF and 16SR). Standard PCR was carried out to amplify DNA from each colony and the amplified product was identified by DGGE analysis. By tallying the identity of 50 clones in each library, we obtained an approximate estimate of the proportions of fish DNA present in each sample. The fish component of the daily diet comprised of herring (50%), smelt (36%) and salmon (14%). If the prey DNA in the clone libraries were present in identical proportions to the mass of the prey items in the diet, the expected range in the proportions estimated by our approach is quite wide simply due to sampling variability. For a random sample of 50 drawn from a multinomial distribution with the proportions 50%, 36% and 14%, the observed proportions have a 95% chance of falling in the range 50% ± 13.9%, 36% ± 13.3% and 14% ± 9.6%, respectively {calculated using the formula $p \pm 1.96\sqrt{(p(1-p)/50)}$ where p is the true proportion}.

Data analysis

For statistical tests, each DNA extraction was treated as an independent sample. Statistical differences in PCR detection rates were evaluated by chi-squared contingency table tests for comparisons between (i) the different prey species, (ii) the blended samples and the subsamples, and (iii) the samples collected during the basic diet equal and unequal meal feeding regimes. Chi-squared tests were also carried out to evaluate whether our estimates of the proportions of fish DNA were consistent between scat samples and whether these estimates were consistent with the proportions of fish mass in the diet. Statistical tests were performed using the R Foundation for Statistical Computing version 1.9.1 alpha software (R Development Core Team 2004).

Results

DGGE separation of PCR products

The DGGE conditions that allowed separation of 16S1F and 16S2R clamp PCR products for the six prey species were a linearly increasing 30–70% denaturing gradient (with 100% denaturants being 40% formamide and 7 M urea) in a 7.5% polyacrylamide gel. PCR products amplified from the fish DNA using the primers 16SfishF and 16S2R clamp were separated on a 6% polyacrylamide gel that also contained a linearly increasing 30–70% denaturing gradient (Fig. 3a). The running temperature for both was 56 °C and the 16 cm gels were run at 70 V for 8 h. Initial runs separating bands amplified from genomic DNA of the prey species identified two alleles for herring. These alleles ran further than bands from any other species on the gel and both were scored as herring in subsequent analysis.

Detection of prey DNA in feeding trial scat samples

The average mass of scat used in the DNA extractions was 79 ± 29 mg. The initial PCR tests were performed on DNA from five scat samples using the 16SF and 16SR primers (universal approach, Fig. 2). Amplification from each produced a single band which did not migrate with any of the prey bands on the DGGE gel. The five amplification products were cloned and four clones from each were sequenced. All 20 sequences matched perfectly with a Steller sea lion sequence from GenBank (NC004030). An additional 50 clones were screened using DGGE in an attempt to identify DNA from prey which could potentially be present at low level in these PCR products. Each of these clones also originated from sea lion DNA.

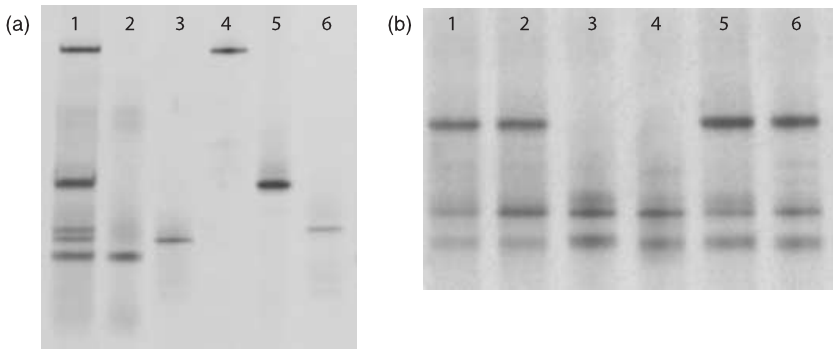

Fig. 3 Denaturing gradient gel electrophoresis of 16S mtDNA fragments amplified from fish prey species fed during the feeding trial. (a) PCR products amplified from genomic DNA template: five species mix (lane 1), Pacific herring (lane 2), smelt (lane 3), pollock (lane 4), sockeye salmon (lane 5) and capelin (lane 6). (b) PCR products amplified from DNA extracted from scat. Four samples amplified salmon, smelt and herring DNA (lanes 1, 2, 5, 6) and two samples amplified only smelt and herring (lanes 3, 4).

To overcome swamping by the predator's DNA, all further prey detection attempts were carried out using the group-specific approach (Fig. 2), which excluded sea lion DNA from amplification. When the sea lions were fed the basic diet (i.e. excluding the scats collected when animal 1 was fed unequal meals as well as those collected within 48 h of the pulse species being fed), a total of 108 samples (27 blended and 81 subsamples) were collected from 27 scats. Using squid-specific primers, PCR amplification of DNA extracted from these samples gave an overall percent frequency of detection (PFD) of 94% — with squid DNA being detected in at least one of the samples from every scat. Using the fish-specific primers, the PFD of fish DNA was 97%. The PFD levels for the individual fish species scored on DGGE gels (Fig. 3b) were 94% for herring, 87% for salmon and 92% for smelt. As with the squid, DNA from each fish species was detected in at least one sample from every scat. The detection levels of the four prey species in the basic diet were not significantly different from one another ($\chi^2 = 0.4307$; d.f. = 3; P value = 0.9338). The PFD values were significantly higher for the blended scat samples (98%) compared with the subsamples (90%) ($\chi^2 = 6.5$; d.f. = 1; P value = 0.011); results are summarized in Table 2.

Table 2 Frequency of detection of prey DNA in scat samples collected during the basic diet feeding regime of the feeding trial (108 samples collected from 27 scats). This summary excludes results from scats collected within 48 h of the sea lions being fed pulse diet items and results from scats collected when diet was being fed in unequal meals

	Blended sample	Subsample	Total
Squid	100% (27/27)	93% (75/81)	94% (102/108)
Herring	100% (27/27)	93% (75/81)	94% (102/108)
Smelt	96% (26/27)	90% (73/81)	92% (99/108)
Salmon	96% (26/27)	84% (68/81)	87% (94/108)
Total	98% (106/108)*	90% (291/324)*	92% (397/432)

*Difference between blended and subsamples $\chi^2 = 6.48$, d.f. = 1, P value = 0.011.

In scat samples collected from Animal 1 while it consumed unequal meals, the overall PFD was 66% vs. a PFD of 95% when it received the basic diet with meals of equal composition (Table 3). This difference in detection level was highly significant ($\chi^2 = 52.46$; d.f. = 1; P value < 0.001) and reflects patchy prey detection (which roughly corresponds to meal composition), not an increase in the number of scat samples failing to produce any PCR products. Smelt was the only species included in both daily meals during the 14-day unequal meals feeding regime. In scats collected over this period, smelt had a PFD of 86% compared with 61% for herring, 68% for salmon and 50% for squid. Results from the pulse prey feedings (pollock fed for a single day and capelin fed for a single day on two occasions) showed that both pollock and capelin were only detected immediately following their inclusion in the diet and their detection was limited to scats collected within 48 h of consumption (Table 4).

Control samples

DNA extracted from the control scat samples (collected from sea lions fed only herring or pollock) produced no visible

Table 3 Frequency of detection of prey DNA in scats collected from a sea lion while being fed the same basic diet in either: (1) two daily meals of equal size and species composition (60 samples from 15 scats) or (2) two daily meals of unequal size and species composition (28 samples from 7 scats). Results exclude the scats collected within 48 h of the animal being fed pulse diet items

	Equal meals	Unequal meals
Squid	95% (57/60)	50% (14/28)
Herring	95% (57/60)	61% (17/28)
Smelt	95% (57/60)	86% (24/28)†
Salmon	97% (58/60)	68% (19/28)
Total	95% (229/240)*	66% (74/112)*

†Smelt was included in both meals, remaining species fed exclusively in meal 1 or meal 2; *difference between equal and unequal meals $\chi^2 = 52.45$, d.f. = 1, P value < 0.001.

Table 4 Summary of the PCR detection results in days following inclusion of pulse species (pollock or capelin) in the diet for a single day. Each shaded block represents a scat sample and symbols show the presence (+) or absence (–)of the DNA in the blended sample and three subsamples tested

	Day 1	Day 2	Day 3	Day 4	Day 5
Pollock	– – – +	+ + – –	– – – –		– – – –
Capelin 1	+ + + +		– – – –	– – – –	– – – –
Capelin 2	+ + – –	+ + + +		– – – –	
	+ + + +				

PCR products with the squid primer set and the fish primer set produced single bands on the DGGE gels which matched the expected prey species. All extraction blanks were negative.

Time course samples

The two scat samples that were subsampled over an extended period initially had detectable DNA present from each of the four prey species in the basic diet. The first scat was sampled from 0 to 8 days with most samples being taken over the first two days. The second scat was sampled every few days over an 18-day period. In both samples detection failed simultaneously for all prey markers (between days 5 and 8 for the first scat and between days 2 and 7 for the second scat). We tested all samples with the 16S1F and 16S2R conserved primer set to determine if the decomposing scats had produced chemicals with a strong inhibitory effect on PCR amplification. These primers produced PCR products for all of the subsamples, indicating amplification was possible from the templates where detection of prey DNA had failed.

Unknown samples

Using the blinded procedure we tested 12 scat samples for the presence of six potential prey items. DNA was extracted from each sample only once. Two of the 12 samples failed to produce any PCR products, 28 prey items were detected in the remaining 10 samples (Table 5). Subsequent comparison of the genetic test results with known prey species in the diets indicated that we identified 100% of the species eaten in eight of the scat samples and identified the major diet component but missed other minor prey species in two samples (Table 5). No false positives were obtained in this analysis.

Quantitative estimates

Analysis by PCR/cloning detected all of the fish prey species in the five scat samples collected when the sea lions were fed two equal meals per day. The proportional estimates from these scats were consistent with each other ($\chi^2 = 9.47$; d.f. = 8; P value = 0.305) even though there was considerable variation (herring ranged from 54% to 72%, smelt ranged from 12% to 28% and salmon ranged from 16% to 28%); results summarized in Fig. 4a. Only one of the estimates from these five scat samples was consistent with the proportions of fish mass in the diet (i.e. had a chi-squared value greater than 0.05). Herring and salmon tended to be over-represented while smelt was under-represented in the clone libraries (Fig. 4a).

The proportional estimates were much more variable for the three scat samples collected when the daily diet was fed in two unequal meals. The two libraries that were produced from individual scats, showed that the proportions of fish DNA matched the composition of individual meals better than it did the overall daily diet (Fig. 4b). The daily proportion of herring was grossly underestimated in one library and salmon was completely absent in the other. Only the proportion of smelt (which was fed in both meals) was estimated reasonably well. The quantitative estimate obtained from the clone library produced by mixing DNA from seven of these patchy scats did provide an estimate more in line with the overall proportions of prey items in the diet (Fig. 4b).

Discussion

Although previous studies have amplified prey DNA from vertebrate scat using PCR, our study reports the first results from a controlled feeding trial looking at detection

Table 5 Results of blind PCR tests preformed on 10 scat subsamples. Symbols indicate presence or absence of DNA marker in scat/presence or absence of prey species in diet, incongruent results are shaded. Results are not shown for two scat samples which produced no PCR products

	Scat samples									
	1	2	3	4	5	6	7	8	9	10
Pollock	–/–	–/–	–/–	–/–	+/+	–/–	–/–	–/–	–/–	–/–
Salmon	+/+	+/+	+/+	+/+	–/–	–/+	+/+	+/+	–/+	–/–
Capelin	+/+	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–
Smelt	+/+	+/+	+/+	+/+	–/–	–/+	+/+	+/+	–/+	–/–
Herring	–/–	+/+	+/+	+/+	–/–	+/+	+/+	+/+	+/+	+/+
Squid	+/+	+/+	+/+	+/+	–/–	–/+	+/+	+/+	–/+	–/–

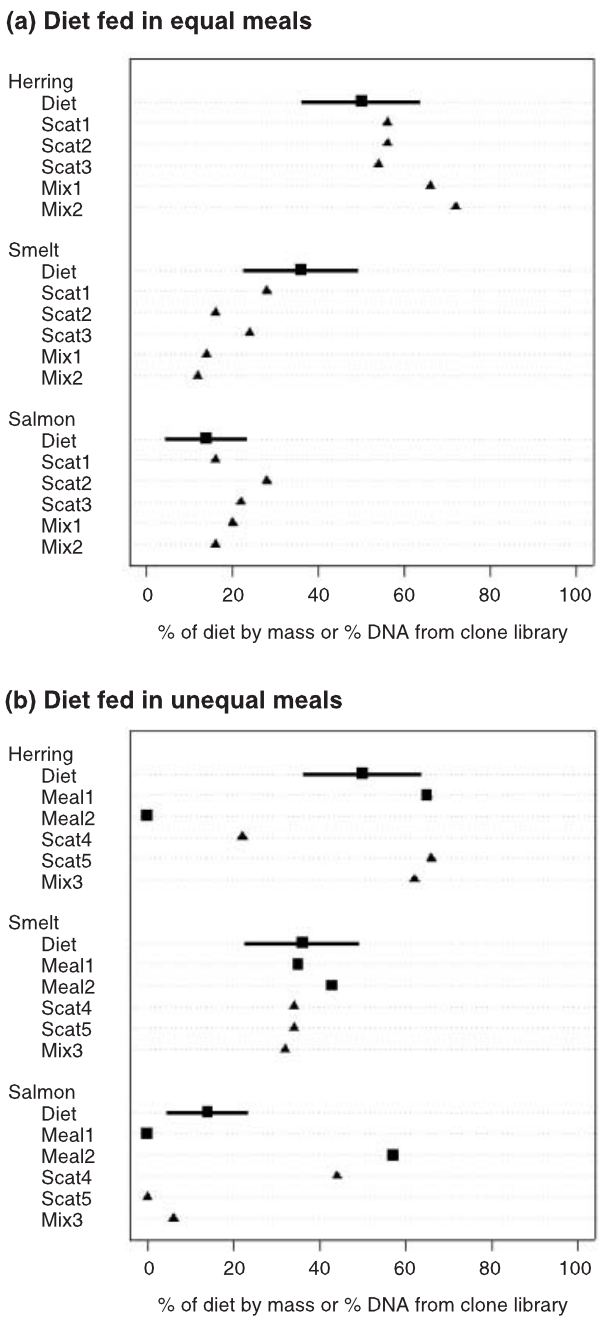

Fig. 4 Dot plots showing estimates of the proportions of fish species in diet obtained through quantification of DNA in clone libraries. Points show the proportion of each fish species by mass in diet (■) and DNA proportions in clone libraries (▲). Bars represent the 95% probable range of estimates (due to sampling error) if DNA proportions are equivalent to mass proportions (see text for details). Clone libraries represent DNA from single scat samples (Scat1–5) or mixtures of DNA from seven scats (Mix1–3). (a) Results from scats collected when the two daily meals were identical in composition. (b) Results from scats collected when the two daily meals differed in composition. Proportion of each fish species by mass in the two meals is shown (■)

of DNA from several prey species. We initially attempted to use a single PCR test to simultaneously amplify DNA from all prey items present in each scat sample. This approach has advantages, primarily because primer binding and PCR conditions will be consistent for all prey species, and the laboratory analysis is minimized. However, primers which are conserved among the target prey species usually also amplify DNA from the predator by necessity. This was a serious problem in our study—by direct screening of PCR products generated using universal primers we were only able to detect sea lion DNA in the scat samples. Predator DNA was expected to be prevalent as a previous study found that nearly one-third of the PCR products generated from fin whale scats (using metazoan-specific primers) matched the fin whale DNA sequence (Jarman *et al*. 2004). However, the absolute dominance of sea lion DNA was unexpected and reinforced the need to actively exclude the predator DNA from analysis. This can be accomplished by designing PCR primers targeting evolutionary cohesive groups of prey that will not amplify predator DNA (see Jarman *et al*. 2004), or it can be achieved using subtractive screening methods (such as predator DNA specific restriction enzyme digestion or subtractive hybridization).

We chose to employ group-specific primers that targeted short regions of fish-specific (mitochondrial) and squid-specific (nuclear ribosomal) DNA. PCR products from the five fish species were separated using DGGE. Using this approach, our prey DNA detection success rates were very high (close to 100% for samples taken from scat samples that had been blended). Detection success rates for squid and sockeye salmon, which were fed as a relatively small percentage of the daily diet, were equivalent to rates for the more abundant diet items (smelt and herring). We did find that the detection of prey DNA was less consistent in samples taken from a small distinct part of individual scats compared with samples that were taken from blended scat. This finding suggests that DNA from different prey species is not distributed evenly within a single scat and has implications for the development of sampling protocols. Our results also demonstrate that prey DNA from different meals consumed on the same day is not well mixed among scats, implying that the DNA in each scat represents the prey species consumed over a short time interval. Analysis of scats produced after the inclusion of novel pulse prey items in the diet showed that detection of the novel prey DNA is restricted to scats produced within 48 h of consumption. While these results need verification with other species of prey (and predator species), they indicate that prey DNA in scat samples can be reliably detected through PCR analysis and this can provide fine resolution data on recently consumed prey. The limited time that prey DNA can be detected after ingestion is a constraint shared to some extent by hard part analysis (Tollit *et al*. 2003); this is a serious limitation for studies of marine mammals that

forage long distances from sites where scats can be collected. Estimates of diet over longer periods may have to be obtained from alternative techniques such as analysis of fatty acids (Bradshaw *et al*. 2003; Iverson *et al*. 2004) or by using animal-borne video systems (Bowen *et al*. 2002).

Using DGGE to separate DNA markers from multiple fish species worked satisfactorily in our captive feeding trial but there were a few limitations. First, the presence of sequence diversity within the herring that were used in the experimental feedings resulted in two markers coming from this single prey item. This feature could confuse interpretation of results in a field application where more interspecific prey diversity might be expected. Second, we found a bright heteroduplex band formed between capelin and smelt. This band migrated near the pollock-sized band and could be accounted for in our controlled study (because pollock and capelin were not used as feed simultaneously) —but again, this could cause difficulties in a field application. We suggest that DGGE should only be applied when the diversity of the prey targeted by the PCR tests is limited and well defined, otherwise cloning and sequencing is probably a better option (e.g. Jarman *et al*. 2004; Kasper *et al*. 2004).

PCR-based detection methods have several limitations in situations where target DNA is present in low amounts and the quality of samples is poor (Taberlet *et al*. 1999). The problem most likely to be encountered in the analysis of prey DNA in scat is the production of false positive and/or false negative results. Cooper & Poinar (2000) outlined procedures for working with ancient DNA to help prevent the occurrence of false positives (amplification of small amounts of contaminant DNA when target DNA is absent in the sample). To apply all these procedures to studies of prey DNA from even moderate numbers of scat samples would be unfeasible. Fortunately, molecular analysis of prey DNA in contemporary scat samples is not quite as extreme as ancient DNA research. Physical isolation of workspace for pre-amplification steps, proper use of negative control amplifications, verification of the reproducibility for a subset of samples and some cross-validation with independent hard part analysis are minimum precautions that need to be carried out to allow confidence in positive results from field studies (Taberlet *et al*. 1999).

The production of false negative results (failure of amplification when target DNA is or was present in the sample) could be due to a number of reasons. These include degradation of the DNA present in the sample, failure of the DNA extraction or failure of the PCR amplification. In our study, the known diet and high prey DNA detection rates allowed us to rule out the occurrence of large numbers of false negatives. However, in studies where the diet is unknown, monitoring the incidence of false negatives is extremely difficult. An indication of the potential frequency of false negatives in molecular scatology studies can be obtained by looking at the amplification success rate of predator mtDNA from scat (Table 6). These data show that it can be difficult to amplify predator mtDNA from scats even though this target is expected to be ubiquitous and, based on our results, present in higher quantities than prey DNA. It is also obvious from these data that the frequency of negative results obtained in different studies varies considerably, with field-based studies tending to have a higher incidence of negative results compared with captive animal studies.

Completely eliminating false negatives in DNA-based diet studies is unlikely to be possible. However, there are ways to reduce the occurrence of this type of error. Obtaining fresh scat samples is of primary importance. We found that in unpreserved scat samples, prey DNA was no longer detectable after 5 to 7 days; this indicates that while scats do not have to be collected immediately after defecation, they should be as fresh as possible. The identification and exclusion of samples of very poor quality can be performed by prescreening of DNA extractions (e.g. Morin *et al*. 2001) or through the use of internal positive controls (i.e. primer sets which target prey groups expected to be represented in all scats). When focusing on good quality scats, the

Table 6 Percentage detection of predator mtDNA from studies carrying out PCR analysis of DNA from mammalian scat

Detection of predator mtDNA (%)	Sample size	Species	Reference
70	20	Harbour and grey Seals	Reed *et al*. (1997)
80	20	Black bear	Wasser *et al*. (1997)
90*	50	Sun bear	Wasser *et al*. (1997)
100	23	Mustelid species	Hansen & Jacobsen (1999)
59	34	Felid and fox	Farrell *et al*. (2000)
53	163	Marten and fox	Davison *et al*. (2002)
90*	30	Marten	Davison *et al*. (2002)
84	130	Wolf	Lucchini *et al*. (2002)
88*	300	Brown bear	Murphy *et al*. (2003)
77	128	Fox, wolverine	Dalén *et al*. 2004

*Captive animals.

number of false negatives obtained can be reduced by processing several samples per scat. In the current study, we failed to detect some prey DNA in scat subsamples collected when the sea lions were being consistently fed the basic diet. However, when we pooled data from the four samples analysed from each scat, DNA from all prey species was identified in every scat.

Estimating not only prey diversity but also relative amounts of prey eaten is the goal of many pinniped diet studies. This information can be used in conjunction with estimates of the predator species' energetic requirements and prey energy density to obtain overall consumption estimates (e.g. Olesiuk 1993; Winship & Trites 2003). Presence/absence data from hard part studies has been used to estimate the relative frequency of occurrence of different prey in the diet (Sinclair & Zeppelin 2002). Genetic data seems suited to this type of analysis because large numbers of samples can be screened once appropriate tests have been developed (although the potential occurrence of false negative results must be carefully considered; see previous discussion). The heterogeneous distribution of prey DNA within a scat and the short detection period of prey DNA are also advantageous in this type of study because the detection of prey in different scat samples will likely represent independent observations (see Tollit *et al*. 2003 for discussion).

Estimates of the relative proportions of different prey species in the diet can be improved through the reconstruction of prey biomass. In studies that rely on recovering and identifying hard parts, this is accomplished by estimating the number and size of prey consumed based on counts and measurements of hard parts recovered in scat. These estimates are biased because the percentage of hard parts recovered from different prey species varies considerably and the size of hard parts is often reduced due to digestion (Tollit *et al*. 1997; Bowen 2000). In captive feeding trials, the recovery rates for fish otoliths range from 0 to 89% and otolith digestion results in the underestimation of fish length by 16–51% (Tollit *et al*. 1997). Correction factors have been developed to account for these biases (Harvey 1989; Tollit *et al*. 1997), but final estimates remain limited by wide confidence intervals.

Genetic analysis of scats could potentially provide biomass estimates if the amount of DNA from each prey species is proportional to the mass of the prey in the diet. Even if it is only possible to classify prey biomass present in each scat into a few categories using DNA quantification (e.g. < 20%, 20–50%, 50–80%, > 80%), these data would allow a substantial improvement in estimates of overall proportions of the prey in diet compared with simple presence and absence data (S. Jarman, unpublished). Our quantitative estimates of the proportions of fish prey DNA in scats did provide a rough estimate of the proportion of the fish present in the meals fed during the trial. Averaged over a number of samples, this level of accuracy would provide useful data on diet composition and will likely provide better quantitative estimates than obtained from hard part analysis under some scenarios (Da Silva & Neilson 1985; Jobling 1987). By pooling DNA from several 'patchy' scats collected when a sea lion was fed two meals with different prey composition, we obtained a composite picture of overall diet rather than of individual meals. This sample-averaging approach could be useful for reducing the number of samples that need to be analysed. We did observe some directional biases in the amount of DNA coming from different prey species. This could be due to a methodological factor such as PCR bias (Suzuki & Giovannoni 1996), or it could be due to species-specific differences in mtDNA copy number, cell density or DNA survival during digestion. Presumably correction factors similar to those used with hard part dietary data could be developed and applied. Based on these initial quantitative results, further assessment of the ability of DNA amounts in scats to estimate proportional diet composition seems warranted. The clone-screening quantification technique we used is straightforward to apply; however, it is labourious (precluding the analysis of a large number of samples) and allows only end-product analysis. Real-time PCR quantification could be a more productive approach because it will allow rapid simultaneous quantification of DNA from multiple prey species. Real-time PCR will also allow quantitative comparisons between prey species targeted by different PCR tests (e.g. the amount of fish compared with squid in the current study); this type of comparison was not possible using the clone library approach we employed.

The use of molecular scatology to study diet has the potential to provide new insight into the diet of vertebrate predators. More accurate taxonomic identification of food remains in scats can be obtained (Hofreiter *et al*. 2000; Purcell *et al*. 2004) and employing genetic methods has obvious benefits in cases where soft-bodied prey or prey with fragile bones are suspected to be an important part of the diet. The technique also provides a means to carry out an independent dietary analysis of scats. Comparison of genetic and hard part data sets should help clarify the errors associated with each method. Measures of diet diversity and studies focusing on small groups of prey species are currently feasible using genetic techniques. However, genetic markers will need to be characterized for many prey species if the approach is to be widely applied, and several aspects of the methodology will need further development before a complete picture of diet can be constructed through molecular scatology.

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All of the authors are actively involved in studying the diet of large marine predators. Bruce Deagle is examining the ability of DNA-based methods to reconstruct diet for his doctoral thesis within Mark Hindell's group at the University of Tasmania. The current project was supported by and stemmed from the ongoing work in this field by Nick Gales and Simon Jarman, from the Australian Antarctic Division. Dominic Tollit's research interests primarily focus on examination of the diet and prey selection in pinnipeds through hard-part and fatty acid signature analysis. Andrew Trites is Director of the Marine Mammal Unit at the University of British Columbia and has broad research interests in marine mammals of the North Pacific.
