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RESEARCH ARTICLE

Combining DNA and morphological analyses of faecal samples improves insight into trophic interactions: a case study using a generalist predator

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Abstract The diet of pinnipeds is most commonly inferred from morphologically diagnostic remains of prey in their scats. Although this method can generate quantitative estimates of diet simply, important prey types may not always be detected. DNA-based methods improve detection of prey in scats, but they are not quantitative. While some studies have combined morphological and DNA-based methods, these have only assessed prey that are represented by their hard remains in scats. To overcome this bias, we apply molecular and morphological analyses to the soft and hard portions of faecal samples respectively, to estimate the diet of lactating Antarctic fur seals (*Arctocephalus gazella*) on Heard Island. The diet of this population is of particular interest because it is expanding rapidly and may rely to some extent on mackerel icefish (*Champsocephalus gunnari*), which are subject to commercial fisheries. Based on results from morphological analysis and likely important prey types, we tested for DNA remains of *C. gunnari*, myctophids and squid in faecal samples. The proportion of samples ($n = 54$) yielding no dietary information was reduced from around 25.9% using either method alone, to 9.3% when combined. Detection of all prey types tested for was notably improved by integrating molecular and morphological data. Data from either method alone would have underestimated the number of animals consuming *C. gunnari* by around 25.7%. Detection of multiple prey types in samples increased from 9.3% when using morphological analysis only, to 33.3% when using DNA only, to 46.3% when using both methods. Taken in isolation, morphological data inferred that individual seals consume either *C. gunnari* or myctophids, probably foraging in separate locations characteristic of those prey. Including molecular data demonstrated that while this may be true of some individuals, many other seals consume a mixed diet of at least *C. gunnari*, myctophids and squid. This new approach of combining DNA-based and morphological analyses of diet samples markedly increased the number of samples yielding dietary information, as well as increasing the amount of information attained from those samples. Our findings illustrate the broad potential of this technique to improve insight into trophic interactions in marine ecosystems.

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Introduction

Marine mammals are major consumers at multiple trophic levels and may therefore exert significant influence on the structure of some marine ecosystems. For example, Antarctic fur seals (*Arctocephalus gazella*) are generalist predators and are widely distributed in the Southern Ocean, breeding predominantly on islands south of the Antarctic Convergence (Reid et al. 2006). *A. gazella* females raise a single pup over a 4-month lactation period, during which they alternate time ashore suckling their pup with bouts at sea foraging (Doidge et al. 1986). The maternal foraging range is constrained by the pup's requirements and the mother's physiological limits, so hunting tends to be

concentrated on a relatively small area (Green 1997; Bonadonna et al. 2001; Boyd et al. 2002). The impact of intense predation in space and time on a marine community may be considerable (Boyd et al. 2002), which has led to lactating *A. gazella* being the focus of many investigations into predator–prey relationships, interactions with fisheries and monitoring the marine environment (Reid and Arnould 1996; Boyd and Murray 2001; Lea et al. 2002a).

The diet of breeding female *A. gazella* on Heard Island is of particular interest because this population is increasing rapidly and may rely to some extent on the most abundant of the low Antarctic icefish species, *Champsocephalus gunnari*, which have been subject to commercial fisheries since 1997 (Green 2005; Kock 2005a). Breeding of *A. gazella* on Heard Island was first noted by the presence of two pups in 1963 (Budd and Downes 1969). Since then, suitable habitat has increased due to significant retreat of glaciers and the breeding population is currently rising at an estimated 11.5% per year, with 1,278 pups born in the 2003–2004 season (Green 2005). There are few published accounts of the diet of *A. gazella* on Heard Island, none of which investigate the diet of nursing females. These studies found that males consumed a fish diet dominated by pelagic myctophids, with *C. gunnari* also a major component at times (Green et al. 1989, 1991, 1997).

Stocks of *C. gunnari* have decreased substantially over most of their range in the last 20 years. While overfishing is a major cause, these declines are not fully understood, and other proposed reasons have included the effects of predation, large variations in recruitment, and changes in environmental conditions (Everson et al. 1999; Kock and Everson 2003; Kock 2005b; Reid et al. 2005). The distribution of *C. gunnari* is patchy and the few reliable areas of high density aggregations in the Heard Island region are likely to be targeted by both fisheries and *A. gazella* (Williams and de la Mare 1995). Based on previous studies in the region, other important prey of breeding female *A. gazella* at Heard Island are likely to be myctophids, and possibly squid (Green et al. 1989; Cherel et al. 1997; Lea et al. 2002a).

As direct observation of feeding is rare, the diet of pinnipeds is most frequently inferred from morphologically identifiable remains of prey in their scats, due to the ease of sample collection and analysis compared to other indirect methods (e.g. stomach lavage, lethal sampling, stable isotope and fatty acid signature analyses). Although not the only diagnostic structure, sagittal otoliths are most commonly used to identify fish taxa, numbers and sizes, as they are relatively simple to classify and are the most consistently useful structure across fish species. Cephalopod mouthparts and exoskeleton remains are used to identify cephalopod and crustacean prey respectively (Cottrell et al. 1996; Tollit et al. 2003).

However, often a large proportion of scats do not contain diagnostic otoliths or squid beaks (e.g. Green et al. 1991; Acuna and Francis 1995; Lake 1997; Fea et al. 1999). This is because otoliths may be eroded beyond identification or fully digested, squid beaks tend to accumulate in the stomach and are regurgitated, and the excretion of prey hard parts over time is uneven (Pierce and Boyle 1991; Casper et al. 2006). This may result in an underestimation of the importance of certain taxa as prey, particularly fish with fragile otoliths, and squid. For example, cephalopods constitute a key group in the pelagic food web of the Southern Ocean and are a vital food source for albatrosses (*Diomedea* spp.), king penguins (*Aptenodytes patagonicus*) and southern elephant seals (*Mirounga leonina*, Rodhouse et al. 1992, 1996; Cherel and Weimerskirch 1999). Consequently, squid might be expected to be consumed regularly by *A. gazella*, but the occurrence of squid beaks in fur seal scats is frequently low (e.g. Doidge and Croxall 1985; Green et al. 1989; Osman et al. 2004).

In response to these inadequacies of morphological scat analysis, a DNA-based approach to identifying prey has recently been applied to marine vertebrate systems. This method relies on recognizing DNA sequences unique to particular prey taxa in diet samples (reviewed in Symondson 2002; Sheppard and Harwood 2005). Prey DNA can be identified from hard part and amorphous remains in these samples (Jarman et al. 2002; Purcell et al. 2004; Kvitrud et al. 2005; Parsons et al. 2005). Captive feeding experiments on *Arctocephalus* spp. (Casper et al. 2007) and Steller sea lions (*Eumatopias jubatus*, Deagle et al. 2005) found that prey DNA were detected in scats up to 48 h after ingestion. This time scale is comparable to the occurrence of otoliths in scats (Orr and Harvey 2001; Staniland 2002; Casper et al. 2007).

Genetic technology is currently limited, at best, to semi-quantitative assessment of pinniped diet. In a study on *E. jubatus* fed constant proportions of three fish species, Deagle and Tollit (2007) found that the absolute amounts of prey DNA were highly variable between samples, but concluded that semi-quantitative estimates were reasonably accurate. Consequently, some workers have combined the benefits of both DNA-based analysis (improved prey detection) and morphological analysis (simple quantitative evaluation of prey). This approach has been restricted to molecular identification of salmonid otoliths and bones that are not diagnostic morphologically, which are then enumerated (Orr et al. 2004; Kvitrud et al. 2005). While this has been valuable, prey identified this way are still biased towards those that are represented by their hard remains in scats.

To overcome this dependency, we advance this integrated approach by applying DNA and morphological analyses to the amorphous and hard portions of faecal

samples respectively. Further, we combine these analyses to assess the consumption of multiple prey types by a generalist predator, namely, lactating *A. gazella* on Heard Island. Our aims were to (1) compare the trophic relationships inferred by each method used separately, and combined, and (2) assess the importance of *C. gunnari* and squid as prey.

Materials and methods

Sample collection

The study was carried out at Spit Bay, Heard Island (53.11°S, 73.73°E), from 20 December 2003 to 25 February 2004. Faecal samples were collected from 54 lactating *A. gazella* on their return to the breeding colony from a foraging trip. Just prior to this trip, these seals had been instrumented with telemetry and data logging devices to record aspects of their foraging behaviour; these data will be presented elsewhere. Following capture and gas anaesthesia (Gales and Mattlin 1998), the instruments were removed and faecal material was collected via enema (Staniland et al. 2003). Enemas were used because it was important to collect dietary information corresponding to these foraging trips, and also because scats were notably rare in the breeding colony throughout the study period.

The watery component of material retrieved from each enema was decanted and discarded. The remaining portion was preserved in 70% ethanol, double bagged (15 × 23 cm Nasco Whirl-Pak®), stored outside in the shade, and then refrigerated on return to Australia in early March 2004. Mean outside temperatures during the study period were 4.47 ± 0.09°C, (n = 432; Bureau of Meteorology, Australia, unpublished). The hard part and amorphous portions of the samples were separated for morphological and molecular analyses respectively. Each sample was poured into a plastic tray and hard remains removed, washed in distilled water and stored dry. The remaining material was replaced into its original storage bags and used for DNA analysis. To prevent cross-contamination, all sorting items were washed clean of visible organic matter with water, washed with 10% household bleach, and then rinsed with water between each sample.

Morphological analysis

Digital images were taken of all otoliths, and otolith length and width measured using a Leica MZ9.5® microscope and SigmaScan Pro Version 5.0.0 Image Analysis© software. Otoliths were identified using references (Hecht 1987; Williams and McEldowney 1990; Reid 1996), and by comparison with otoliths removed from fish collected on a research voyage (HIPPIES[1]), which overlapped temporally and spatially with the foraging activities of the animals from which the faecal material was collected (Gales et al. 2005). The level of erosion of all otoliths was classed as 1 (intact margins and good medial relief; sulcus well defined), 2 (margins and medial relief flattened; sulcus visible), 3 (identifiable to species, genus or family by general shape; medial relief and sulcus poorly defined or absent) or 4 (unidentifiable). Correction factors of 10 and 20% were applied to measurements of class 1 and 2 otoliths respectively (Reid 1995), which were then used to estimate standard length and mass of prey from allometric equations (Adams and Klages 1987; Williams and McEldowney 1990).

The energetic contribution of each prey type to diet was estimated by multiplying the mean mass of individual prey, its energy density, and the number of all identifiable otoliths (i.e. classes 1, 2 and 3). For this purpose, *Gymnoscopelus* otoliths that could only be identified to genus (class 3) were allocated to species in the same proportion that classes 1 and 2 *Gymnoscopelus* otoliths had been identifiable to species. Otoliths of the myctophids *Gymnoscopelus nicholsi* and *G. bolini* could not be reliably distinguished from each other. Similarly, otoliths of the channichthyids *Champsocephalus gunnari* and *Channichthys rhinoceratus* could not be discerned apart. These similarities were evident both in otoliths from enema samples and in otoliths from intact fish trawled on HIPPIES. As the number of *G. nicholsi* and *C. gunnari* caught on HIPPIES were at least an order of magnitude greater than the number of *G. bolini* and *C. rhinoceratus* caught respectively (R. Williams et al., unpublished), formulae for *G. nicholsi* and *C. gunnari* were used to estimate prey size for these respective groups (Williams and McEldowney 1990). Energy density values were used from fish caught on HIPPIES (S. M. Robinson, unpublished, following methods in Tierney et al. 2002), except for *G. fraseri* (Lea et al. 2002b).

Molecular analysis

DNA extraction

DNA was extracted from faecal material using the Ultra Clean™ Fecal DNA Isolation Kit (MO BIO Laboratories, Inc.). A 300 µL aliquot of each sample was centrifuged for 5 s at 10,000×g. The supernatant was poured off, and the sediment resuspended in 100 µL Bead Solution from the kit by vortexing at medium speed for 5 min. Manufacturer's instructions were then followed using 100 µL of the resulting

[1] Heard Island Predator–Prey Investigation and Ecosystem Study.

mixture. Extraction blanks (no faecal material) were included to check for cross contamination. To provide positive controls and to confirm specificity of primers in PCRs, DNA was extracted from 15–20 mg tissue of various taxa using the Ultra Clean™ Tissue DNA Isolation Kit (MO BIO Laboratories, Inc.).

Design and testing of primers

Based on hard remains of prey recovered from faecal samples, PCR primer sets were designed to detect Nototheniodei (sub-order including Nototheniidae and Channichthyidae) and Myctophidae. As squid may be important prey but poorly detected by morphological analysis, primer sets to detect squid were also used. Positive tests for Nototheniodei DNA were interpreted as consumption of *C. gunnari*. The only other members of this sub-order occurring with reasonable abundance in the Heard Island region are *Lepidonotothen squamifrons* and *Dissostichus eleginoides* (Williams and de la Mare 1995). While *L. squamifrons* appear important in the male *A. gazella* diet at times (Green et al. 1989, 1991. 1997), no otoliths from either of these fish species were found in our samples.

We used a nested PCR approach because the quality of DNA in our samples was poor. With low quality template, nested PCRs are often successful in increasing amplification of target sequences (Roux 1995; Deagle et al. 2003). Use of nested PCRs precluded semi-quantitative analysis as used in Deagle and Tollit (2007), because confidence intervals for quantitative PCR (qPCR) data are disproportionately large when initial template copy numbers are low (Peccoud and Jacob 1996), and these errors are magnified during the second round of a nested PCR. The squid primers (Deagle et al. 2005; Casper et al. 2007) and the external Nototheniodei primer pair (Jarman et al. 2004) had been developed in previous studies. An internal primer for the Nototheniodei assay and all primers for detection of Myctophidae were developed for this study (Table 1).

Primers were designed by an alignment of all sequences available for the target taxon in GenBank in early 2005 as well as sequences from closely related non-target taxa. Alignments were made with ClustalX (Thompson et al. 1997) and primers designed by eye. A BLAST (Altschul et al. 1990) search for short sequences was used to confirm that, of the sequences available in GenBank, the primer sequence only occurred in the target taxon. An initial assessment of primer pair quality was made using Primer3 (Rozen and Skaletsky 2000). Primer annealing temperatures were optimised by temperature gradient qPCR on target taxon DNA. This was followed by a combination of qPCR to test priming efficiency and gel electrophoresis to ensure that the correct fragment sizes were amplified. The specificity of the primer sets for their target taxon was checked by applying them to representatives of a wide range of groups also found in the Heard Island region, such as Amphipoda, Chondricthyes Copepoda, Euphausiacea, Isopoda, Mysidacea and Ostracoda, as well as Channichthyidae, Myctophidae and squid. Testing of non-target taxa from Arripidae, Carangidae, Clupeidae, Mullidae, Octopoda, Salmoniformes, Scombridae and Sillaginidae was also performed. No cross amplification was detected in any of these cases, confirming the specificity expected from the in silico design. Primer sets, the DNA region targeted and the expected product sizes are presented in Table 1.

Table 1 PCR primers used and product sizes

Primer name	Sequence 5′–3′	Approximate product size (bp)	Target (gene:taxon)
Squid28SF[*, a]	CGCCGAATCCCGTCGCMAGTAAAMGGCTTC		Nuclear 28S rDNA:squid
Squid28SR[*,a]	CCAAGCAACCCGACTCTCGGATCGAA	180	Nuclear 28S rDNA:squid
Squid28S2F[#, b]	CCTTCGGGACGWGTGGCGCA		Nuclear 28S rDNA:squid
Squid28S2R[#, b]	CCGTCGCTCGCCGTCCGCACC	100	Nuclear 28S rDNA:squid
Noto16SF[*, #, c]	CCCTATGAAGCTTYAGACRTA		mtDNA 16S: Notoheniodei
Noto16SR[*, c]	CCTTGTTGATAWGGTCTCTAAAA	275	mtDNA 16S: Notoheniodei
Noto16S2R[#, d]	AGGAGTTGTACTCCYGTTCCAC	150	mtDNA 16S: Notoheniodei
Mycto12SF[*, #]	CCGTTCAACCTCACCACTTC		mtDNA 16S:Myctophidae
Mycto12SR[*]	GGGTCAGAAAATGTAGCCCATC	170	mtDNA 16S:Myctophidae
Mycto12S2R[#]	GCRCACCTCGACCTGA	90	mtDNA 16S:Myctophidae

[*] External and [#] internal primers in nested PCRs

Source: [a] (Deagle et al. 2005)

[b] (Casper et al. 2007),

[c] (Jarman et al. 2004),

[d] B. E. Deagle (unpublished)

Real-time PCRs were carried out on a Chromo 4™ Continuous Fluorescence Detector and analysed using Opticon Monitor™ software (MJ Research). Amplification of DNA was visualised using the fluorescent dye SYBR Green® I (Molecular Probes) that selectively binds to double-stranded DNA (dsDNA). Although dyes such as SYBR Green® I provide a simple generic method for product detection, all dsDNA products are detected. Use of this dye to identify target sequences therefore depends on the specificity inherent in the amplification primers (Wittwer et al. 1997; Morrison et al. 1998). To provide evidence that amplifications from faecal DNA represented target product, we incorporated a melting curve analysis into the cycling protocol for internal primer sets. The melting temperature of nucleic acids is affected by factors such as length, G+C content and presence of base mismatches (Anon 2004). As fluorescence of SYBR Green® I is related to the amount of dsDNA, the melting temperature of a PCR product corresponds to a sudden decrease in fluorescence. If primers are specific, the melting temperature of product amplified from faecal DNA should be similar to product amplified from target prey tissue DNA (positive control). Establishing that the product has only one major melting temperature also provides confidence of primer specificity. We further confirmed target product by repeating nested PCRs on all samples that had amplified (omitting the melting cycle), and compared product size with the positive control using 1.5% agarose gel electrophoresis.

PCR amplification

Each 20 μL test reaction contained DNA template combined with 2 μL 10× AmpliTaq PCR Gold buffer, 2 μL 25 mM $MgCl_2$, 1 μL 1× SYBR® Green I, and 0.2 μL each of 100× BSA, 10 mM dNTPs, 10 mM forward primer, 10 mM reverse primer and AmpliTaq Gold (Applied Biosystems). Template for external PCRs consisted of 2 μL DNA extracted from faecal material for Myctophidae and Notothenioidei tests, and 1 μL for squid tests. PCR product from these reactions provided the template for internal PCRs, 2 μL for Myctophidae and Notothenioidei, and 1 μL for squid. Cycling conditions for PCRs were 95°C for 10 min, followed by X cycles of 94°C for 5 s, Y°C for 30 s, 72°C for 20 s, then 72°C for 10 min, where $X = 35$ and 30, 45 and 35, and 30 and 35 cycles, and $Y = 65$ and 59°C, 59 and 63°C, and 61 and 62°C, for external and internal PCRs of Myctophidae, Notothenioidei and squid respectively. Melting curves were resolved after completion of internal PCRs by reading fluorescence every 0.2°C from 55 to 96°C. All PCR runs included a positive control to confirm suitable reaction conditions, and a PCR blank and a DNA extraction blank to check for cross-contamination. Aerosol-resistant tips were used for preparation of all reactions and open PCR products were handled in a separate laboratory. All PCRs were prepared under a laminar flow hood using UV sterilized equipment and consumables. All samples classified as positive for target DNA using real-time PCR amplification curves were supported by melting curve and gel electrophoresis results, confirming that each PCR product had the expected size and sequence.

Data analysis

Prey taxa consumed as determined by morphological and DNA-based analyses were compared by calculating the proportion of faecal samples in which a particular prey type was detected (frequency of occurrence; FO), using each method. Prey type in this context refers to Myctophidae, Channichthyidae and squid. The non-parametric Wilcoxon rank-sum procedure was used to test for significant differences between samples containing only channichthyid or myctophid remains with respect to number of otoliths per sample, the proportion of unidentified otoliths per sample, and the date of sampling. Date was analyzed as a continuous variable. The standard error (SE) is given as a measure of variability about the mean.

Results

Diet

Qualitative analysis

Of the 54 faecal samples, 15 did not contain diagnostic hard remains of prey and 13 tested negative for DNA of all 3 target taxa, but only 5 of these were the same samples (Table 2). Similarly, the level of detection of channichthyids was comparable using otoliths (27 samples) and DNA (28), but only 18 of these were the same samples. Myctophids were identified in fewer samples using otoliths (16) than DNA (26), 11 of which were the same. One squid beak was present, while squid DNA was identified in 8 other samples. Using both morphological and genetic analyses, channichthyids were detected in 37 samples, myctophids in 31, and squid in 9 samples. Multiple prey taxa were detected in 5 samples using morphological analysis, 18 samples using genetic analysis and 25 samples using both methods (Table 2). Date of sampling had no effect on the occurrence of faecal material containing only myctophid or channichthyid remains, as determined by otoliths alone ($\chi^2_1 = 0.354$, $P = 0.552$, myct.: $n = 12$, chann.: $n = 22$), DNA alone ($\chi^2_1 = 0.139$, $P = 0.709$, myct.: $n = 10$, chann.: $n = 13$) or both ($\chi^2_1 = 0.602$, $P = 0.437$, myct.: $n = 9$, chann.: $n = 15$).

Table 2 Frequency of occurrence (FO) of three taxa in lactating *A. gazella* faecal samples ($n = 54$), as determined by morphological and genetic analyses

FO (*n*)	Ch. DNA (13)	My. DNA (10)	Ch. and My. DNA (10)	Ch. and Sq. DNA (2)	My. and Sq. DNA (3)	Ch., My. and Sq. DNA (3)	No DNA (13)
Ch. otoliths (22)	6	2	4	1	0	2	7
My. otoliths (12)	0	4	4	0	2	1	1
Ch. and My. otoliths (4)	4	0	0	0	0	0	0
Ch. otoliths and Sq. beaks (1)	1	0	0	0	0	0	0
No otoliths (15)	2	4	2	1	1	0	5

Ch. channichthyid, *My.* myctophid, *Sq.* squid

Quantitative analysis

A total of 590 otoliths, one upper squid beak and two *Themisto gaudichaudii* amphipods were recovered. Squid and amphipods were excluded from further quantitative analyses. Fifteen percent of otoliths were unidentifiable. The rest were from myctophid and channichthyid fish (Table 3). Except for four samples, identifiable otoliths in each sample were either all myctophids ($n = 12$) or all channichthyids ($n = 23$; Table 2). One sample (from seal FF28) contained 186 myctophid otoliths. Excluding this sample, the mean number otoliths/sample was 10.63 ± 1.56 (median = 7, range = 1–41, $n = 38$). There was no significant difference in the number of otoliths/sample (excluding FF28), or in the proportion of unidentifiable otoliths/sample (including FF28), between myctophid and channichthyid dominated samples ($\chi^2_1 = 0.238$, $P = 0.626$ and $\chi^2_1 = 0.643$, $P = 0.423$ respectively). Myctophid otoliths were dominated by the *G. nicholsi*/*G. bolini* complex and *G. fraseri*, but due to their greater size, the former prey contributed 85.5% of the energy provided by the myctophid portion of the diet. Channichthyid otoliths all belonged to the *C. gunnari*/*C. rhinoceratus* complex. Based solely on morphological analysis, myctophids and channichthyids contributed an estimated 42.3 and 57.7% to the diet in terms of energy respectively (Table 3). The result was similar when energetic contributions to diet of these taxa were calculated using molecular and morphological analyses, by allocating 10.63 fish per sample where a target taxon was detected with either method (based on mean otoliths per sample), and using mean mass and energy density values of *G. nicholsi* and *C. gunnari* (Table 3; myctophids 43.5%, channichthyids 56.5%).

Discussion

Trophic relationships

DNA-based analysis identified each target prey taxon, as well as multiple prey types in the same sample, more frequently than morphological analysis. As detection of prey types using each method was not always in the same samples, combining data from both sources enhanced

Table 3 Otolith types found in lactating *A. gazella* faecal samples, with estimated prey sizes and energetic contributions to diet

Taxon	Total otoliths	Mean standard length (mm) (range, *n*)	Mean mass (g)	Energy density wet mass (kJ g^{-1})	Total energy (kJ) (% contribution to diet)
Gymnoscopelus nicholsi/ G. bolini	120	134.3 ± 1.2 (100.0–162.6, 94)	29.5 ± 0.8	[a]8.41	35,672 (36.1)
G. fraseri	98	73.0 ± 0.7 (56.8–105.5, 90)	4.5 ± 0.2	[b]10.2	5,416 (5.5)
Gymnoscopelus sp.	44				
Protomyctophum bolini	23	56.0 ± 1.6 (30.2 – 64.5, 23)	2.4 ± 0.2	[a]6.83	383 (0.4)
P. normani	1	57.1	2.6	[a]4.99	13 (<0.1)
Electrona antarctica	4	82.0 ± 3.4 (71.9 – 86.3, 4)	7.6 ± 0.9	[a]8.62	259 (0.3)
Krefftichthys anderssoni	1	31.9	0.3	[a]8.10	2 (<0.1)
Total Myctophidae	291				41,745 (42.3)
Champsocephalus gunnari/ Channichthys rhinoceratus	211	206.3 ± 2.7 (130.0–315.3, 94)	67.2 ± 3.5	[a]4.02	57,002 (57.7)
unidentifiable	88				

Energy density:

[a] S. M. Robinson (unpublished data),

[b] (Lea et al. 2002b)

interpretation of the diet further. The proportion of samples yielding no dietary information was reduced from around 25.9% using each technique alone, to 9.3% when they were combined. The proportion of samples with multiple prey types increased from 9.3% when using hard parts only, to 33.3% when using genetics only, to 46.3% when using both methods (Table 2).

Based on morphological analysis alone, it would appear that individual seals predominantly consumed either myctophids or channichthyids, and that squid comprised a negligible portion of the diet (Table 2). As there was no evidence of a shift in prey type as the season progressed, this could then be interpreted as consumption of either myctophids or channichthyids resulting from individuals foraging in different locations. For example, *G. nicholsi* is one of the most abundant myctophid species in the Southern Ocean occurring in the open ocean and over continental shelves and oceanic banks (Linkowski 1985; Hulley 1990), while *C. gunnari* have a fragmented distribution around islands and on shallow banks in the Heard Island region (Williams and de la Mare 1995).

Based on molecular analysis alone, it would appear that consumption of both myctophids and channichthyids by individuals was common, and that the contribution of squid to diet may be important. These trophic interactions are even more evident when data from molecular and morphological analyses are integrated (Table 2). While many individuals may have consumed either myctophids or channichthyids, combined data show that a significant proportion of seals consumed multiple prey types.

The fact that scats were rarely found at the breeding colony implies that the last meals taken by females before hauling out are likely to represent diet where they spent most of their time foraging. Further, if prey DNA and hard part remains in scats represent consumption within the past 48 h (Deagle et al. 2005; Casper et al. 2007), then the enema samples from our study probably correspond to prey ingested closer to 48 h than just prior to collection. This further restricts the ranges of time and space within which many female *A. gazella* found and consumed both channichthyids and myctophids, and sometimes squid. Samples where only channichthyids or myctophids were detected may reflect foraging by these individuals in locations where channichthyids and myctophids do not occur together. Alternately, detection of only channichthyid or myctophid consumption during less than 48 h of a foraging trip does not necessarily mean that those individuals did not consume a more diverse diet over the duration of the entire foraging trip (mean = 8.0 ± 0.5 days, R. M. Casper et al., unpublished). This latter interpretation is supported by results from HIPPIES, where trawls coinciding with areas that female *A. gazella* spent most of their time foraging were dominated by *C. gunnari*, *G. nicholsi* and squid (R. Williams et al., unpublished). These net samples also support the assumption that positive tests for Nototheniodei and Myctophidae DNA in enema samples predominantly represent primary ingestion of *C. gunnari* and *G. nicholsi* respectively.

The question arises as to why DNA and otoliths of a taxon were not always detected in the same samples. There are a number of likely contributing factors. Each method requires a different minimum quality for detection and these do not inevitably occur together, i.e. otoliths need to have retained sufficient morphological integrity to allow identification, while target sequences of prey DNA need to be present in sufficient amounts to effect a positive test result. Secondly, the solid and liquid portions of digesta flow at different rates through the gastrointestinal tract (Mårtensson et al. 1998), so even if otoliths occur in a sample, DNA of that species is not necessarily present in the amorphous portion of the sample, and vice versa. Also, the excretion of otoliths tends to be in pulses while that of prey DNA is more consistent over time, so faecal samples are more likely to contain prey DNA than otoliths (Casper et al. 2007). This pulsing could also explain the lack of mixing of myctophid and channichthyid otoliths in samples. Although both prey types were consumed by some individuals within a limited time frame, these seals may have encountered and consumed groups of each taxon separately within this period.

Another consideration is the possibility of false DNA positives resulting from secondary ingestion. It is unlikely that this occurred as a consequence of prey consumed by *C. gunnari* or *G. nicholsi*. In the Heard Island region, *C. gunnari* reach sexual maturity at 230–270 mm, and animals younger than this (i.e. those taken by seals in this study, Table 3) prey on *T. gaudichaudii* and small euphausiids (Kock 2005a). While older fish may take myctophids, in an 8-year study on the Kerguelen Plateau sampling *C. gunnari* up to 400 mm, only *Euphausia vallentini* and *T. gaudichaudii* were common prey (Duhamel 1991). The diet of *G. nicholsi* consists of euphausiids, hyperiid amphipods, copepods and mysids (Hulley 1990). Further, there was no evidence from stomach contents of myctophids or *C. gunnari* caught on HIPPIES of consumption of each other or squid (R. Williams et al., unpublished). There is a paucity of information on the diet of Southern Ocean squid. Some data are available for *Moroteuthis ingens*, which do consume myctophids (Jackson et al. 1998; Phillips et al. 2001). It is possible that some positives for myctophid DNA may be due to secondary ingestion by squid, but this is unlikely to be common as both myctophids and squid were detected in as many samples as both *C. gunnari* and squid were (n = 6 each, Table 2). Importantly, there is no evidence that detections of squid DNA are not due to primary ingestion.

A further issue to consider is whether invertebrates such as euphausiids and amphipods were overlooked as important

prey. The exoskeletons of *E. superba* are poorly digested by *A. gazella* (Staniland 2002), and *E. superba* are well represented in the scats of *A. gazella* populations in the Atlantic sector of the Southern Ocean (Reid et al. 2006). It is therefore not likely that invertebrates with chitinous exoskeletons in the Heard Island region, such as *E. vallentini* and *T. gaudichaudii*, would be poorly represented by morphological analysis if they were important prey of *A. gazella*. There are, however, other potential prey such as chondrichthyians that could be overlooked by morphological analysis. It may be worth testing for DNA of these taxa in the future.

Champsocephalus gunnari as prey

Morphological analysis offers a simple means of describing prey consumed, allowing size overlap with the *C. gunnari* fishery to be assessed. The estimated standard length of *C. gunnari* consumed by *A. gazella* during our study (206 ± 3 mm; Table 3) was generally less than the minimum legal total length allowed to be taken by fisheries (240 mm, CCAMLR 2003). That is, there was little competition in sizes of *C. gunnari* taken by female *A. gazella* and legal fisheries in 2004. Although *A. gazella* do not appear to consume prey longer than 240 mm often (Green et al. 1989; Lea et al. 2002a; Reid et al. 2005), this is unlikely to be the result of selective foraging. A multiyear cycle of *C. gunnari* occurs on the Kerguelen Plateau, on which Heard Island lies, usually dominated by a single cohort (Duhamel 1991, 1995; Williams et al. 2001; Kock and Everson 2003). In May 2004, the population in the vicinity of Heard Island was dominated by a very strong 2+ year class, with a length distribution similar to that taken by the seals (Davies et al. 2004).

Integrating morphological and molecular data improves the dietary information on which management decisions can be based. Combined analyses suggest that *C. gunnari* was the major dietary component of female *A. gazella*, occurring in nearly 70% of samples and comprising nearly 60% of their energetic requirements (Tables 2, 3). Taken in isolation, each method would have underestimated the proportion of the population consuming *C. gunnari* by around 25.7%. Morphological data alone inferred that many seals may rely exclusively on *C. gunnari*. While this could be the case for some individuals, integrated data show that many seals consume a more diverse diet.

Female *A. gazella* are flexible foragers and optimise their foraging time budget to maximise the rate of energy transfer to their pups under all conditions (Arnould et al. 1996; Boyd 1999; Staniland and Boyd 2003). It has also been shown that female *A. gazella* individuals tend to return to similar foraging locations, at least when these patches have been profitable (Bonadonna et al. 2001; Staniland et al. 2004). It is not known how the likely increasing predation pressure from *A. gazella* will affect the *C. gunnari* population at Heard Island, nor how female *A. gazella* respond during years when the abundance of *C. gunnari* is low or when the main cohort is large enough to be legally fished. Longer term studies are warranted to assess these issues.

Squid as prey

Squid taxon and size can be identified from lower beak morphology, but this was not possible in our study as we recovered only one upper beak. Molecular testing revealed that squid are probably important prey, occurring in 16.6% of samples. While this does not indicate the mass consumed, Southern Ocean squid may be a significant source of energy for *A. gazella* due to their high lipid content, which often exceeds 40% (Phillips et al. 2001; McArthur et al. 2003; Phillips et al. 2003). Morphological analysis underestimated the number of animals consuming squid by 88.9%, providing a strong case for routine testing of *A. gazella* scats for squid DNA.

Conclusions

This is the first study to assess the diet of a free-ranging generalist using DNA-based analysis of the amorphous portion of faecal material together with conventional morphological analysis. This approach markedly increased the number of samples yielding dietary information, as well as increasing the amount of information attained from those samples. This is because tests for prey DNA were independent of the presence of morphological remains of prey in those samples. Detection of *C. gunnari*, myctophids and squid were all notably improved, and the number of animals consuming each of these prey would have been significantly underestimated based on morphological data alone. Morphological analysis implied that individuals mostly consume a monotypic diet, but inclusion of molecular data suggests that a large proportion of seals consume a more diverse diet. As diet samples are likely to reflect prey consumed within the final 48 h of a foraging trip, it is possible that individuals for whom only one prey type was detected may have also consumed a mixed diet over the course of the entire trip. While morphological analysis is the most accessible method of assessing diet, the molecular approach used here is reasonably simple and clearly adds value to the data obtained. The relative merits of morphological and DNA analyses of pinniped scats are discussed in detail in Casper et al. (2007). Our findings indicate considerable potential for this integrated technique to improve insight into trophic relationships in any system where diet samples can be analysed by both morphological and DNA-based methods.

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