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PERMANENT GENETIC RESOURCES ARTICLE

Proportion of prey consumed can be determined from faecal DNA using real-time PCR

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Abstract

Reconstructing the diets of pinnipeds by visually identifying prey remains recovered in faecal samples is challenging because of differences in digestion and passage rates of hard parts. Analysing the soft-matrix of faecal material using DNA-based techniques is an alternative means to identify prey species consumed, but published techniques are largely nonquantitative, which limits their usefulness for some applications. We further developed and validated a real-time PCR technique using species-specific mitochondrial DNA primers to quantify the proportion of prey in the diets of Steller sea lions (*Eumetopias jubatus*), a pinniped species thought to be facing significant diet related challenges in the North Pacific. We first demonstrated that the proportions of prey tissue DNA in mixtures of DNA isolated from four prey species could be estimated within a margin of ~12% of the percent in the mix. These prey species included herring *Clupea palasii*, eulachon *Thaleichthyes pacificus*, squid *Loligo opalescens* and rosethorn rockfish *Sebastes helvomaculatus*. We then applied real-time PCR to DNA extracted from faecal samples obtained from Steller sea lions in captivity that were fed 11 different combinations of herring, eulachon, squid and Pacific ocean perch rockfish (*Sebastes alutus*), ranging from 7% to 75% contributions per meal (by wet weight). The difference between the average percentage estimated by real-time PCR and the percentage of prey consumed was generally < 12% for all diets fed. Our findings indicate that real-time PCR of faecal DNA can detect the approximate relative quantity of prey consumed for complex diets and prey species, including cephalopods and fish.

Keywords: diet, faeces, prey quantity, real-time PCR, Steller sea lion

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Introduction

Identifying the type and quantity of prey species consumed by predators is critical for assessing trophic interactions, and the factors that influence the dynamics of animal populations over time (Pimm 2002; Trites 2003; Winship *et al*. 2006). However, noninvasive reconstruction of diets is challenging if foraging cannot be directly observed. This is particularly true in marine mammals.

The diets of pinnipeds (sea lions, seals and walruses) have traditionally been described from the presence of prey species and diagnostic hard part remains retrieved from their stomachs (Frost & Lowry 1980; Prime & Hammond 1987; Scheffer 1928), and more recently from faecal samples for seals and sea lions (Merrick & Loughlin 1997; Sinclair & Zeppelin 2002; Tollit *et al*. 2007, 2009; Trites *et al*. 2007). However, accurately resolving and quantifying the prey species consumed from faecal samples have been limited by the differences in digestion among the prey species eaten, and by the lack of hard parts in some prey (Bowen 2000; Tollit *et al*. 2007, 2009).

Stable isotope, fatty acid and DNA techniques have all been developed more recently to overcome the limitations associated with reconstructing diets from hard part analysis. Stable isotopes from prey are assimilated into whiskers, teeth and other tissues and provide trophic-level information over varying timescales, but are unable to provide species-level resolution (Baylis *et al*. 2009; Hobson *et al*. 1996, 1997; Sinisalo *et al*. 2008). In contrast, quantifying the proportions of fatty acids of different prey species deposited in pinniped blubber has had mixed success as a result of variability in prey fatty acid

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signatures, high sensitivity to fatty acid-specific calibration coefficients and unassessed issues associated with rates of assimilation of prey fatty acids (Meynier 2009; Nordstrom *et al.* 2008; Tollit *et al.* 2007). DNA-based techniques using faecal samples, on the other hand, have been more successful at identifying the presence of specific species (Casper *et al.* 2007; Marshall *et al.* 2010; Tollit *et al.* 2009). One advantage of DNA-based techniques over fatty acid and stable isotope techniques is that they can identify the species of prey consumed with greater confidence and resolution (Deagle *et al.* 2005; King *et al.* 2008; Tollit *et al.* 2009) and their application can be considered a more universal technique.

Developing an effective method of diet reconstruction is particularly important for Steller sea lions because of drastic population declines in the Gulf of Alaska and Aleutian Islands (Trites & Larkin 1996; Winship & Trites 2006). Steller sea lions range from northern California to British Columbia and Alaska, and across the Aleutian Islands to Russia and northern Japan, with the largest concentration historically residing in western Alaska (Aleutian Islands and Gulf of Alaska) (Loughlin *et al.* 1992). The western population of Steller sea lions has declined since the late 1970s and is classified as endangered, while the eastern population has increased (3% per year, SE Alaska to California). Regional differences in the quality or quantity of prey species consumed is hypothesized to underlie the divergent dynamics of the two sea lion populations (DeMaster & Atkinson 2002; Nordstrom *et al.* 2008; Rosen & Trites 2005; Trites & Donnelly 2003; Winship & Trites 2003). The conservation status of Steller sea lions and the difficulty with current methods of diet analysis present a need to develop better methods for estimating prey consumption.

DNA analysis has been used to determine the presence and absence of prey in pinniped diets and has the potential to estimate quantities of prey species consumed (Deagle *et al.* 2005; Deagle & Tollit 2007; King *et al.* 2008). However, only a few studies have attempted to determine the quantities of prey species consumed from the presence of DNA in faeces (Deagle *et al.* 2005, 2009; Deagle & Tollit 2007; Matejusova *et al.* 2008). Two of these studies tested whether diets fed to captive Steller sea lions (*Eumetopias jubatus*) could be reconstructed from test clone libraries (Deagle *et al.* 2005) and real-time PCR (Deagle & Tollit 2007). These studies identified the prey species fed and derived consumption estimates that were within ~11% of the proportions fed after applying correction factors for mitochondrial DNA (mtDNA) density of the prey (amount of mtDNA per gram of tissue) (Deagle & Tollit 2007). Such results are promising, but the study was based on a small number of scats with only three prey species fed in fixed proportions. Further evaluation through additional feeding experiments with larger sample sizes, more prey species and varying proportions in the diet is required before the approach can be applied in field-based studies, because the limitations of quantifying prey species consumed by their DNA are not clearly understood.

The goal of our study was to undertake a comprehensive validation of real-time PCR as a tool for estimating the percentages of prey consumed by Steller sea lions using DNA extracted from faeces. We began by validating that real-time PCR can detect the relative amounts of different prey species in mixes of prey DNA. We then attempted to determine the relative amounts of prey DNA contained in the faeces of captive Steller sea lions fed 11 different diets containing known amounts of four prey species. The diets fed to Steller sea lions consisted of between two and four species, ranging from 7% to 75% contributions to a meal mix (by wet weight). Our study shows that the relative amounts of prey consumed by Steller sea lions can be determined approximately from the amounts of DNA present in their faeces.

Methods

Real-time PCR

PCR primers specific to the mitochondrial genes encoding 16S ribosomal RNA for herring (*Clupea palasii*), eulachon (*Thaleichthyes pacificus*) and rockfish (both *Sebastes helvomaculatus* and *alutus*) and cytochrome oxidase 1 (CO1) for squid (*Loligo opalescens*) were designed using Primer Express Software (v2; Applied Biosystems Inc.). Note that some of the primers we used (Table 1) closely matched to species that were not in our study based on comparisons to all available sequence data (using the NCBI BLAST tool) and were thus only species specific within the context of our study.

We extracted total DNA from previously frozen herring, eulachon, squid and rockfish tissue using the DNeasy Blood and Tissue kit, according to the 'animal tissue' protocol (Qiagen). Total DNA was extracted from 93.6 ± 26.9 mg of homogenized soft-matrix from faeces stored in ethanol using the QIAmp DNA Stool Mini kit (Qiagen), according to the 'Isolation of DNA from stool for human DNA analysis' protocol (Qiagen), as in Deagle *et al.* (2005) and Deagle & Tollit (2007). The only exception was that we eluted DNA in the AE buffer provided with the kit. The amount and quality of DNA present in each extraction were determined using a Nanodrop (ND-1000) spectrophotometer.

All real-time PCR reactions were performed using an ABI 7000 sequence detection system (Applied Biosystems, Inc.). Reaction conditions were one cycle of 50 °C/2 min, 95 °C/10 min, 40 cycles of 95 °C/15 s, 60 °C/1 min, one cycle of 95 °C/15 s, 60 °C/20 s, 95 °C/15 s. Reaction

Table 1 Real-time PCR primers used in this study

Accession number	Target gene	Species	F-forward or R-reverse	Sequence 5′–3′	Amplicon size (number of base pairs))
EU548092	16S	*Clupea palasii*	F	CGCCCACCAATCACGAA	69
			R	ACGTTTGTGCCAGTATCACGTT	
EU548154	16S	*Thaleichthyes Pacificus*	F	GAGAAGACCCTATGGAGCTTTAGACA	79
			R	GGAGTCACAATGTTTTTTCCCTTT	
AF000051	CO1	*Loligo opalescens*	F	TTAGCATCCTCGGCTGTTGA	77
			R	CCAGCATGAGAGAGATTTCTAGATAGG	
EU548166	16S	*Sebastes helvomaculatus*	F	GAGCACCCCCTCCTACAATTAA	74
			R	CGGCATTGCCGGATCTTA	
EU548165	16S	*Sebastes alutus*	F	GAGCACCCCCTCCTACAACTAA	74
			R	CGGCATTGCCGGATCTT	

F and R denote forward and reverse.

volume was 22 μL, containing 4 μmol of each primer and 2 μL of each sample (4 ng total DNA for all reactions except for Feeding Experiment 2, for which 10 ng total DNA was added) with 10 μL SYBR Green Master mix (Applied Biosystems Inc.).

A two-fold serial dilution (containing seven dilutions, and ranging from 4 ng total DNA in the well to 0.0625 ng total DNA in the well) was included on each plate, with DNA taken from the prey species being identified as a template, and species-specific primers were used for amplification. These serial dilution amounts were chosen based on the hypothesized range of concentrations of prey DNA in a faecal sample, as well as practical decisions about the amount of DNA that is necessary to pipet accurately to have a strong standard curve for comparison. We ran this standard curve in triplicate on each plate and used it to calculate the relative quantity of DNA in the unknown samples (which were assayed in duplicate). We obtained r^2 values of ≥ 0.989 for all standard curves and slopes between −2.97 and −3.63, which indicated 90–110% efficiency of the real-time reactions. Using a standard curve approach corrects for the ratio of mitochondrial DNA to genomic DNA (in a total DNA extract) in the tissue when known amounts of total DNA from each prey species are mixed prior to real-time PCR.

Percent of DNA in mixtures of prey DNA

We assessed the sensitivity of real-time PCR to small changes in total DNA added using species-specific mitochondrial DNA primers to amplify DNA that was extracted directly from herring (*Clupea palasii*) and squid (*Loligo opalescens*) and mixed in seven different combinations (%:%) (2:98, 5:95, 25:75, 50:50, 75:25, 95:5, 98:2). We made these mixes by diluting extracted DNA of each species to a common concentration of 2 ng/μL and mixing the two species to the percentages specified above. The final concentration of each mix was also 2 ng/μL. We then used real-time PCR to estimate the total quantity and proportion of species DNA in the mix. In short, by real-time PCR, for each mix we obtained quantities for each prey species separately, summed these values to make a total quantity and then calculated relative percent contribution of each species in the mix.

We extended our validation of the herring and squid mixes to eight additional DNA mixes comprising combinations of herring, squid and two additional species (eulachon *Thaleichthyes pacificus* and rosethorn rockfish *Sebastes helvomaculatus*). These mixes mimicked diets 2–10 in Feeding Experiment 2 (see Diets 2–10 in Table 2). We chose these four prey species because (i) herring is one of the staples of the sea lion diet based on hard part analysis (Sinclair & Zeppelin 2002), (ii) eulachon could be a seasonally important energy-rich food source (Sigler *et al.* 2004), (iii) squid and other cephalopods are not always passed consistently because of the retention of beaks in the stomach and intestines (Bowen 2000; Tollit *et al.* 2006, 2003), and (iv) rockfish is a bony fish like herring and eulachon but it is difficult to distinguish the different species of rockfish consumed by Steller sea lions using diagnostic hard remains.

Feeding trials and sample collection

We used the PCR assay outlined above to estimate the percent of prey consumed by captive Steller sea lions fed known amounts of diets consisting of various mixtures of prey species. The faecal samples we used were collected in two separate feeding experiments: (1) from 12 October 2006 to 7 February 2007, called 'Feeding Experiment 1', and (2) from 16 February 2009 to 28 April 2009, called 'Feeding Experiment 2'. Six female Steller sea lions between the ages of 3 and 6 years old participated in the two studies. They were captured as pups from northern Vancouver Island and studied at the Vancouver Aquarium. All research was conducted under the

Table 2 Percentage by weight of prey species fed to Steller sea lions in Feeding Experiment 2

Diet	Herring	Eulachon	Squid	Rockfish
Diet 1	75	0	25	0
Diet 2	50	0	50	0
Diet 3	50	50	0	0
Diet 4	75	25	0	0
Diet 5	25	75	0	0
Diet 6	25	25	25	25
Diet 7	25	50	0	25
Diet 8	0	50	0	50
Diet 9	25	25	50	0
Diet 10	0	25	0	75

approved University of British Columbia animal care protocol # A07-0413.

Feeding Experiment 1. Faecal samples were collected from four Steller sea lions fed identical diets. Two of the sea lions (F03WI, F03IZ) were 3 years old and two (F00ED, F00YA) were 6 years old at the time of the study. They were housed with one another in different combinations in enclosures consisting of a pool and haulout platform. The animals were fed a diet consisting of four prey species over 12 weeks. By weight, the diet consisted of 64.3% herring, 14.3% eulachon, 14.3% squid and 7.1% rockfish (*Sebastes alutus* and not *Sebastes helvomaculatus* which was used for the prey DNA mix validation described above). Food intake (±0.01 kg) was controlled daily. Faeces were collected regularly over the course of the study, and subsamples of each faeces (soft-remains only) were collected for DNA analysis as in Tollit *et al.* (2009). The weight of each scat was recorded, as was whether it was collected from the haulout or pool.

Feeding Experiment 2. Ten diets were fed in sequence to two 6-year-old Steller sea lions (F03AS and F03RO) (Table 2). Their diets consisted of constant percentages of prey that were usually fed in two meals per day (although not all meals were the same weight over the course of a day or between days). It was necessary to allow the weight of meals to vary to maintain the animals at appropriate body weights throughout the study, and because the analysis was dependant on the percentage of prey in a diet as opposed to the weight of the whole diet, it was possible to vary the weight of meals with no consequence. The sea lions were housed together in an enclosure containing a haulout and pool while faeces were collected. Each of the diets was consumed for about 7 days.

There was a 3-day 'flush period' at the beginning of each feeding regime before faecal samples were collected. During this time, the animals were housed with other animals or in other pools and were fed the same diet as they were for the period of sample collection. At the start of the fourth day, the animals were placed in a cleaned pool (either flushed and cleaned, or netted and vaccuumed). Scat was collected opportunistically from the pools and haulouts, and while training sessions were in progress over the next 4 days. Attempts were made to always collect the entire scat.

Each faecal sample was collected in a ziplock bag, homogenized by hand and weighed. Using a tongue depressor, a portion of each homogenized sample was forced through a 0.5-mm mesh secured with an elastic band over a piece of acrylic pipe. Approximately 3 mL of soft-matrix was then scraped from the underside of the mesh and placed into a faecal collection tube containing 15 mL of 95 % EtOH.

Correction factors for total mitochondrial amount

DNA isolated from faeces contains DNA from prey, DNA from the bacterial flora and fauna of the gut and DNA from the predator itself. In our real-time PCR, we used a different standard curve for each prey species based on dilutions of total DNA (~genomic DNA) as described in the *real-time PCR* section above. Because the ratio of genomic DNA to mtDNA can differ between species (because of differences in mtDNA copy number or genome size), the amount of mtDNA in each standard curve may vary. This means that either the measured prey mtDNA values or the amounts consumed by the animals ('the expected mtDNA values') needed to be adjusted to a common standard curve. We therefore derived a correction factor to adjust the amounts of prey species consumed (by weight) for differences in mtDNA relative to total genomic DNA.

We developed species-specific mtDNA correction factors by estimating the difference in the absolute amount of mtDNA for a given amount of total DNA among the various prey species. DNA isolated from the tissues of the four prey species was mixed in equal parts (25% for each of herring, eulachon, squid and rockfish). We then used an approach similar to that presented in Scott *et al.* (2005) that required selecting a common threshold value for all real-time PCR and using this to calculate PCR efficiency and amounts of mtDNA for each of the four prey species (herring, eulachon, squid and rockfish) according to the following factors:

(1) $Efficiency\ (E) = 10^{-1/slope}$

(2) $Amount\ of\ mtDNA\ in\ sample = E^{-Ct}$

(3) *Amount of mtDNA per total amount of DNA added = amount of mtDNA in sample/total amount of DNA added to sample*

We used our calculated mtDNA correction factors to correct the percentage of prey fed to the animals (expected value by weight) for the ratio of mtDNA to genomic DNA. This generated a new 'expected value' for the amount of mtDNA present in the faeces. We subsequently compared these expected results to the observed amounts of mtDNA in the faeces as estimated using real-time PCR.

Statistical analysis

Statistical analyses were performed using R (R Development Core Team 2008; Data S1). We tested whether the proportions of prey measured by real-time PCR in the 11 diets fed in Feeding Experiments 1 and 2 were over- or under-representations of the proportions expected using a *t*-test. For each species fed (herring, eulachon, squid and rockfish), we regressed the expected proportions against the average proportions observed over all the diets using a weighted regression. The weight given to each diet was inversely proportional to the variance $w = n * \hat{y} * (1 - \hat{y})$, where w equals the weight given to the diet, n equals the number of independent trials (diets in which that prey species was fed), and $\hat{y}$ equals the predicted proportion from the regression. We then performed a one-sample *t*-test to determine whether the slope of the line was significantly different from unity (one).

Results

We estimated the accuracy of real-time PCR for quantifying prey DNA by mixing isolated prey DNA in known quantities and comparing these to estimates generated using real-time PCR (Table 3 and Fig. 1). Overall, we found that real-time PCR determined percentage of DNA present within a margin of ~12%, demonstrating that real-time PCR can be used to quantify DNA that is present irrespective of the species of origin. We then used data from the mix containing 25% of each herring, eulachon, squid and rosethorn rockfish to develop a mtDNA correction factor for differences in mtDNA relative to total genomic DNA among the prey species. We estimated that the amount of mtDNA in each species relative to herring was 1.000 for herring, 0.506 for eulachon, 0.102 for squid and 0.046 for rockfish.

The diet consumed by the Steller sea lions in Feeding Experiment 1 contained 64.3% herring, 14.3% eulachon, 14.3% squid and 7.1% rockfish (by weight), and after applying the above correction factors to these amounts we expected to see relative mtDNA percentages of 87.7% herring, 9.9% eulachon, 1.99% squid and 0.44% rockfish in the DNA from faeces. As shown in Fig. 2, the measured percentage of prey estimated by real-time PCR was 70.9 ± 11.1% herring, 17.5 ± 9.8% eulachon, 8.6 ± 3.7% squid and 2.9 ± 2.5% rockfish. For Feeding Experiment 1, the estimated percentages of prey consumed were within ~17% of the expected values.

We predicted that faecal samples collected from the pool might not be representative of all the prey species that were present because faecal samples are not necessarily homogeneous masses (Deagle *et al.* 2005) and the remains of some prey species may wash away in water. However, average estimated percentages of prey species in the diet did not differ appreciably when samples were collected from the pool or the haulout (Fig. 3).

Table 3 Real-time PCR estimates of prey species in seven mixtures of herring and squid DNA

% in Mix		% Measured	
Herring	Squid	Herring	Squid
98	2	98 ± 0.2	2 ± 0.2
95	5	95 ± 0.3	5 ± 0.3
75	25	77 ± 2.3	23 ± 2.3
50	50	52 ± 3.4	48 ± 3.4
25	75	31 ± 3.3	69 ± 3.3
5	95	7 ± 1.2	93 ± 1.2
2	98	3 ± 0.7	97 ± 0.7

Data are mean ± standard deviation of 10 replicates.

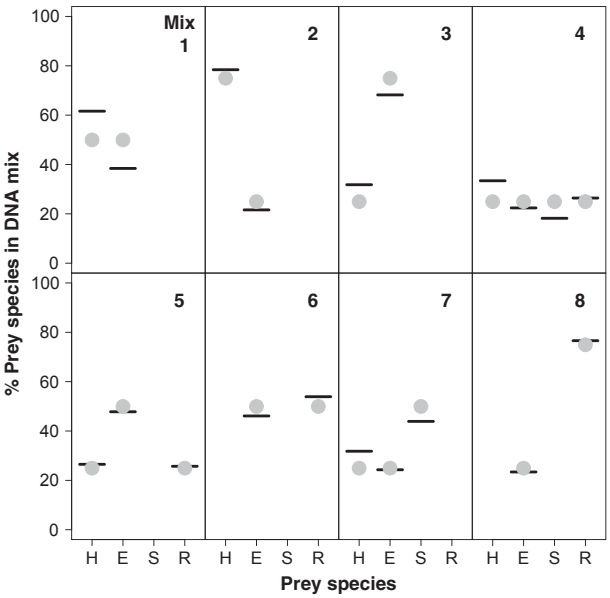

Fig. 1 Real-time PCR estimates of prey species in eight mixtures of prey DNA. Solid dots represent the percent of each prey species' DNA added to the mixture. Bars represent the per cent of each prey species estimated to be present by real-time PCR. Prey species were H-herring, E-eulachon, S-squid, R-rockfish. Each mix was assayed in duplicate.

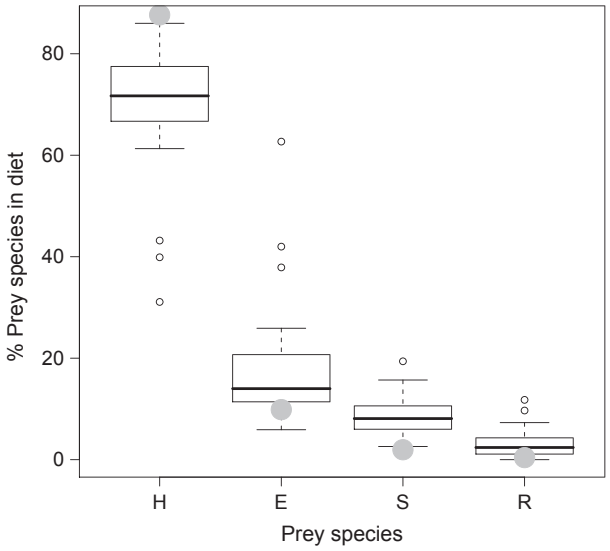

Fig. 2 Real-time PCR estimates of prey DNA in Steller sea lion faeces from a diet of known composition (Feeding Experiment 1). Solid dots represent the percentage of prey that was fed to the animals (by weight and corrected for the ratio of mtDNA to genomic DNA). Boxplots show the median, range and upper/-lower quartiles of the percentage as estimated by real-time PCR for each prey species in the diet H-herring, E-eulachon, S-squid, R-rockfish. $N = 45$ scats.

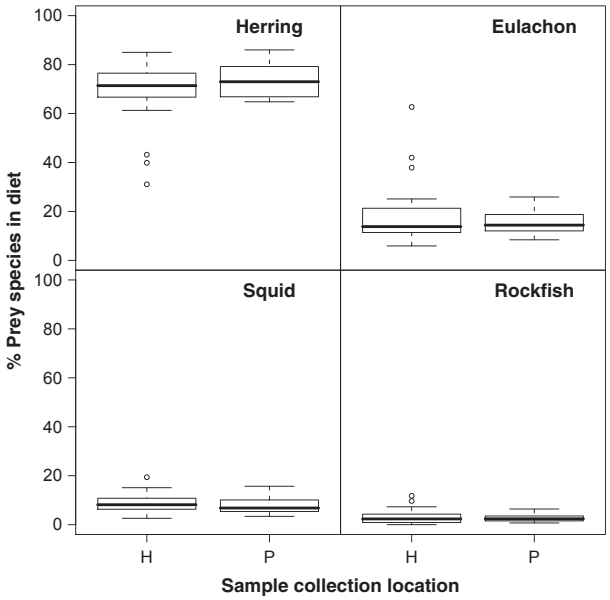

Fig. 3 Real-time PCR estimates of prey DNA in Steller sea lion faeces for a diet of known composition (Feeding Experiment 1), for scats collected from the H-haulout or P-pool of the animals' enclosure. Boxplots show the median, range, and upper/lower quartiles of the percentage estimated by real-time PCR for samples collected from the haulout or pool. $N = 36$ from the haulout and nine from the pool.

The numbers of scats assayed for each of the 10 diets containing herring, eulachon, squid and rockfish in Feeding Experiment 2 (Table 2) were four scats for Diets 2 and 4, five scats for Diets 3, 5 and 7–10, and six scats for Diets 1 and 6. For these diets, the accuracy of percentage estimates was similar to what was seen in both the mixes of DNA from tissue (Table 3 and Fig. 1) and Feeding Experiment 1 (Fig. 2). Overall, the variance of the data decreased at the extreme proportions and the types and proportions of species consumed by sea lions were determined from faecal samples within a margin of ~12% of what was expected (Fig. 4).

To determine whether our real-time PCR technique accurately estimated the proportion of prey in the various diets, we performed a weighted regression of diet proportions estimated by real-time PCR against the expected diet proportion based on weight of food fed, corrected for relative mtDNA amounts (Fig. 5). We used this regression to calculate the slope of the line, and then used a one-sample *t*-test to determine whether this slope was significantly different from a slope of one, which would be expected if real-time PCR provided a close approximation of the proportion of prey in the diet. The slope was not significantly different from one for herring, eulachon or squid, but did differ for rockfish (one-sample *t*-test (two-tailed), $\alpha = 0.05$; n equals the number of independent trials (diets); herring $P = 0.05$, $n = 9$, eulachon $P = 0.30$, $n = 9$, squid $P = 0.94$, $n = 5$ and rockfish $P = 0.003$, $n = 5$; Fig. 5).

Discussion

Real-time PCR is a relatively new molecular method that has proved useful for quantifying the amount of DNA in a sample (Wong & Medrano 2005). In our study, we demonstrated that the technique can be extended for ecologically relevant applications, namely determining the relative amount of prey consumed by Steller sea lions from the DNA contained in their faeces. We successfully reconstructed the diets of Steller sea lions fed 11 different diets from proportions of prey DNA in their faeces. The accuracy of our technique for diet reconstruction from faecal DNA was within 12–17% of the expected quantity after correcting for the relative mitochondrial content of each prey species. We believe that this technique can be applied to samples collected from the field and has many advantages compared to traditional diet analysis techniques.

Real-time PCR diet quantification

Deagle & Tollit (2007) were the first to quantify Steller sea lion diet using real-time PCR. They applied real-time PCR to estimate the proportions of prey in DNA from a

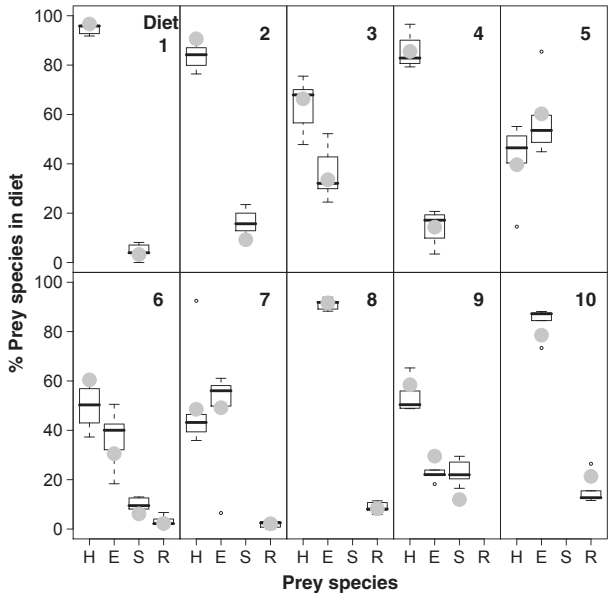

Fig. 4 Real-time PCR estimates of prey DNA in Steller sea lion faeces from 10 diets of known composition. Solid dots represent the percentage of prey that was fed to the animals (by weight and corrected for the ratio of mtDNA to genomic DNA). Boxplots show the median, range and upper/lower quartiles of the percentage estimated by real-time PCR for each prey species in the diet H-herring, E-eulachon, S-squid, R-rockfish. N = 4–6 scats per diet (see text for details).

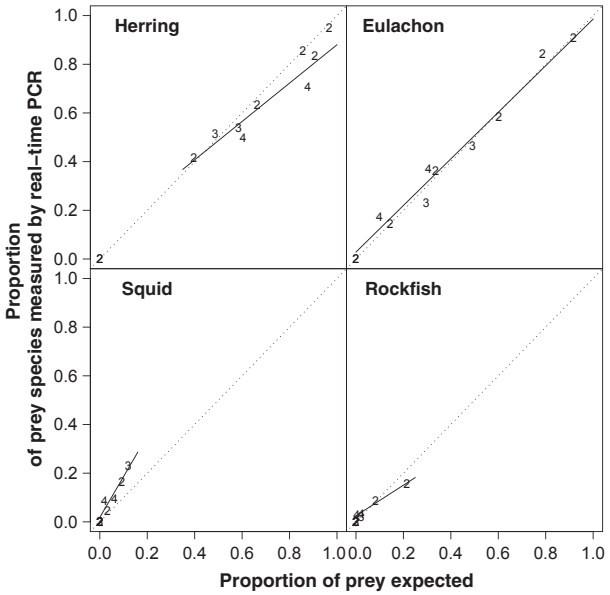

Fig. 5 Weighted regressions of the proportion of prey expected and measured by real-time PCR in Steller sea lion faeces from eleven diets of known composition. Each diet is represented by a number, which shows the number of prey species that were tested in that diet. The solid line represents the best fit line and the dotted line represents a slope of 1. N = 5–9 (see text for details).

tissue mixture homogenate containing herring, salmon and smelt, and from faecal DNA of sea lions fed the same combination of fish. They found that the proportions of prey species estimated by real-time PCR did not exactly match those of the tissue mixture or in the diet (by weight). However, their percentages of prey fell within ~11% of the prey fed when they accounted for the amount of mtDNA per gram of fish tissue. This result for a single mixture of fish demonstrated the potential of real-time PCR but needed to be validated with a wider range and combinations of species.

Our research built on that of Deagle & Tollit (2007) by completing a much more extensive validation of the technique. We fed 11 different diets of four species compared to their single diet combination consisting of three species. Similar to Deagle & Tollit (2007), we found that real-time PCR of mtDNA allowed fairly accurate estimates of diet proportions (within 12–17% as determined by the difference between the proportion of mtDNA expected for a prey species and that estimated by real-time PCR). In our study, this margin of error was likely due to technical error, as estimates of proportions in known mixtures of DNA had a similar level of inaccuracy. We found that variance decreased as proportions became smaller, but recognize that error associated with quantity of infrequently consumed prey may inflate its potential relative importance (e.g., an estimate of 10% for a species representing 5% of diet would mistakenly double the importance of this species).

We also provided a more comprehensive validation of the theoretical accuracy of real-time PCR by analysing mixtures of isolated DNA from four prey species in 15 combinations. Again, these validations indicated that the theoretical accuracy of the technique was within approximately 12% of that expected. One technical difference between the study by Deagle & Tollit (2007) and our research is that they used dilutions of a three-fish plasmid (a circular piece of bacterial DNA that contains the sequences from each of the prey species to be detected by PCR) in their standard curve for real-time PCR. Here, we used a standard curve based on dilutions of genomic DNA. Our results highlight that simple dilution of genomic DNA can provide accurate quantification as long as the ratio of mtDNA to genomic DNA is accounted for (if mtDNA markers are amplified in the PCR). The ease of obtaining genomic DNA rather than producing recombinant plasmids for each species should make future applications of this technique more accessible and practical.

While the raw diet data (Figs 2 and 4) show that the difference between the percentage of prey expected and measured is generally less than 12%, the weighted regressions for squid and rockfish (Fig. 5) suggest that the measured amounts of squid and rockfish are not necessarily

true representations of their contributions to the diet. Squid was generally overrepresented, while rockfish was increasingly underrepresented as the amount of prey consumed increased. This may have been because there was such a small amount of DNA measured for both of these prey. In addition, the amount of DNA expected for squid and rockfish may have been very small once corrected for relative amount of mtDNA, even when they were fed to the sea lions in relatively large amounts (by weight), because squid and rockfish have such a small amount of mtDNA relative to either herring or eulachon (as shown in this study). It may be informative to feed rockfish and squid in a sequence of diets along with other species that have relatively little mtDNA to see if this trend is any different.

The extensive validations that we undertook using many different mixes of DNA and diets fed suggest that real-time PCR is a robust technique for diet quantification. Taken together, the research that we and others have undertaken demonstrates that PCR diet quantification is an effective way to determine the proportions of prey species in diet.

Strengths and weaknesses of real-time PCR relative to traditional hard part analysis

The main advantages of quantifying diet using real-time PCR instead of identifying hard parts are that it is faster for large numbers of samples, more people are able to do it, and it is more repeatable. There are molecular laboratories readily equipped for real-time PCR at most universities and government institutes, and many molecular biologists can perform the protocol we describe. This compares to a handful of people that are qualified to complete hard part analysis, and the even scarcer comprehensive prey reference collections needed for comparisons. Hard part analysis techniques can be subjective relative to molecular methods and require the application of correction factors (as in molecular analysis) to reconstruct the biomass of prey consumed. Studies with captive Steller sea lions have shown that biomass estimates based on counting and measuring diagnostic hard parts can be within 5–12% of the actual diet fed if appropriate correction factors are used (Tollit *et al.* 2007). Calculating correction factors in hard part analysis, however, requires controlled feeding studies to assess intra-specific digestion and passage times (recovery rates) of hard parts. Overall, there appear to be several benefits to using DNA-based diet analysis over traditional diet analysis.

Our data showed that DNA from all of the prey species consumed by the Steller sea lions in Feeding Experiments 1 and 2 were present in the soft-matrix of their faeces. Real-time PCR is therefore not likely to miss prey species because of preferential consumption of parts of prey that do not contain diagnostic hard parts (e.g., salmon or cod bellies) (Tollit *et al.* 2003, 2009; Trites *et al.* 2007). In addition, PCR-based techniques can be more species specific and can therefore quantify species of salmon and rockfish that cannot be identified to species from hard remains. Although the exact sensitivity of our technique is unknown, we detected that rockfish constituted 2.9% of the total prey DNA (or 5.2×10^{-4} ng) in Feeding Experiment 1 and therefore feel that prey present in very small amounts can be quantified. This has significant implications for being able to quantify rare or endangered prey species that are present in very low amounts in predator diets.

DNA analysis and real-time PCR can increase the resolution of prey identification (Tollit *et al.* 2009) and provide reasonable estimates of the proportion of prey consumed (our study) compared to hard parts, but DNA methods cannot determine the sizes of prey eaten, and thus cannot provide information on the total amount of food consumed. Body size (lengths and weights) correlate with the size of beaks, otoliths and other hard remains and can therefore be derived by retaining these diagnostic structures (Tollit *et al.* 2007). Thus, we believe that the optimal dietary analysis should use a combination of molecular and hard part techniques to determine species, composition and size. Developing a model that can take into account the prey sizes obtained from hard remains and the proportional estimates from molecular data to obtain biomass consumption estimates would be a productive next step in diet reconstruction.

All prey items in a predators' diet must be known to accurately assess the relative contribution of each species to the diet, because real-time PCR estimates of prey quantity are represented as proportions. Errors in the estimates of proportions of all prey items will occur if a prey item is missed. For example, in Feeding Experiment 1 we fed the sea lions 87.7% herring, 1.99% squid, 9.9% eulachon and 0.44% rockfish, and estimated from real-time PCR that they had consumed 70.9% herring, 17.9% eulachon, 8.6% squid and 2.9% rockfish. However, we would have incorrectly estimated that they had eaten 60.3% eulachon, 29.7% squid and 10.1% rockfish if we had not included herring (the largest contributor to their diet) in the PCR analysis. In contrast, removing a minor contributor to the diet, such as rockfish in this experiment, would only have had a minor effect, yielding 73.0% herring 18.1%, eulachon and 8.9% squid. It is therefore necessary to have *a priori* knowledge of the possible contents in a faecal sample before real-time PCR is used for analysis.

Potentially, the need for *a priori* knowledge of prey species in the diet can be addressed in field application by first doing a nonquantitative (presence/absence) assessment of many potential prey items in diet samples. For example, Tollit *et al.* (2009) used a group-specific

PCR method in which closely related groups are amplified using PCR while simultaneously excluding the predator's DNA from amplification. In this approach, samples are usually amplified many times with different sets of primers, first targeting large evolutionary groups (i.e., fish or cephalopods) followed by more distinct groups or individual species (i.e., herring or squid). Amplicons can then be separated by DGGE or sequenced to identify the prey species that are present, and real-time PCR primers can be designed to target specific prey or higher-level groups (i.e., fish and cephalopods). An alternative to using DGGE could be to target a few major groups (for example fish and cephalopods) directly with real-time PCR based on a very basic analysis of hard remains. Using the two-step method we outlined above will ensure that all of the prey species that could potentially be in a faecal sample are accounted for and that the quantitative estimates derived from real-time PCR represent their actual contributions to the diet.

Field application

We suggest that determining the proportions of prey consumed by pinnipeds in the wild can be done using scats collected in the field using real-time PCR following five steps. First, all prey species, or groups of prey species, that are in each scat sample need to be identified using a presence-absence PCR technique such as DGGE (described above). Second, mtDNA sequences need to be obtained for the prey types to be targeted by real-time PCR. Third, real-time PCR primers need to be designed and tested on DNA extracted from the tissue of the prey species of interest. Fourth, correction factors need to be calculated for the ratio of mtDNA to genomic DNA using DNA extracted from tissue for the prey species of interest. Finally, the primers and correction factors designed can be used to analyse field samples according to our protocol. Currently it is unclear how many scats need to be analysed to have confidence in proportional estimates using DNA methods. Based on computer models simulating biomass reconstruction (proportion of prey consumed by weight) using hard remains, a minimum of approximately 100 scats provided sufficient precision for temporal or spatial comparisons of grey seal (*Halichoerus grypus*) diet (Hammond & Rothery 1996); however, no formal analysis has been conducted to determine the number of scats required to have confidence in proportional estimates using DNA. In addition, factors such as the length of time that an animal spends on land for breeding, or the distance travelled for foraging can affect temporal and spatial representations of diet. Therefore, it will be important to take life history factors into account before attempting to measure prey consumption. This protocol could be used to determine, for example, the relative amount of specific species of salmon consumed by Steller sea lions, or to assess consumption of other commercially important or endangered fish stocks.

Identifying secondary prey items as part of a predator's diet is a potential concern for all diet studies, but is unlikely to be a significant problem for real-time PCR diet analysis. We added 4 ng of DNA to each PCR, which would have included prey DNA, predator DNA, and DNA from flora and fauna of the predator's gut. Results for Feeding Experiment 1 showed that the average amount of prey DNA (for all four species combined, $n = 45$) was 0.0178 ng—not even 0.5% of the total DNA added. Given that the physical amount of secondary prey is likely to be low relative to primary prey, we believe that the very low amounts of DNA from secondary prey is unlikely to amplify, and in the unlikely event that it does amplify, it will not be detected more often than it is using hard part analysis.

Our study confirms the utility of the DNA-based approach to dietary analysis. The molecular protocol that we developed should be applicable to any predator–prey system if validated with controlled feeding trials as we described. Studies attempting to determine a quantity of prey species in invertebrates and other pinnipeds have shown promise, but often stopped short of being able to deal with differences in DNA density or breakdown during digestion, or they have not been validated in a controlled feeding environment (Deagle *et al.* 2005, 2009; King *et al.* 2008; Matejusova *et al.* 2008; Nejstgaard *et al.* 2008). Cumulatively, these studies show that it should be possible to implement DNA-based approaches to obtain quantitative estimates of prey consumption, but that these approaches may have limitations.

Looking to the future, recent studies have shown that pyrosequencing (and other forms of high-throughput sequence analysis) may be more useful than real-time PCR in some instances (Deagle *et al.* 2009; Valentini *et al.* 2009a,b). For example, Deagle *et al.* (2009) combined group-specific PCR and pyrosequencing to determine population level trends in the diets of Australian fur seals. High-throughput sequencing may soon be more efficient and cheaper than real-time PCR for large-scale and broad analyses. However, real-time PCR will probably have a role in validating that quantitative information can be obtained. Real-time PCR will also have a role in answering questions that are less high-throughput, and in studies looking at specific questions (e.g., the relative proportion of fish versus squid in a diet) or at predator species with a low level of prey diversity in their diet.

Conclusions

Overall, we demonstrated that for a known diet composition real-time PCR is an effective way to quantify prey

consumed by Steller sea lions. Correction factors for the ratio of mtDNA to genomic DNA in prey species can be easily derived and applied to estimate the proportions of prey consumed. This molecular technique works for a range of prey types with different mitochondrial DNA densities and should be applicable to quantify the diets of other predator–prey systems.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Data S1 R routines for analysis and figure presentation of real-time PCR data for Figures 1–5.

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