

DETERMINING THE RELATIVE AMOUNTS OF PREY IN STELLER SEA
LION (*EUMETOPIAS JUBATUS*) DIET USING REAL-TIME PCR

by

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Abstract

Determining 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. Analyzing the soft matrix of faecal material using DNA-based techniques is an alternative means to identify prey species consumed, but published techniques are largely non-quantitative, which limits their applicability. I developed and validated a real-time PCR technique using species-specific mitochondrial DNA primers to quantify the diets of Steller sea lions (*Eumetopias jubatus*). I 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*. I then applied real-time PCR to DNA extracted from faecal samples obtained from Steller sea lions that had been fed 11 different combinations of herring, eulachon, squid and Pacific ocean perch rockfish (*Sebastes alutus*), ranging from 7-75% contributions to a meal mix (by wet weight). The difference between the average percentage estimated by real-time PCR and the percentage of prey consumed was generally less than 12% for all diets fed when percentages of prey consumed were corrected for differences in mtDNA density among the prey items. My findings indicate that real-time PCR can detect the quantity of prey consumed for a variety of complex diets and prey species, including cephalopods and fish.

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Co-Authorship Statement

I completed all of the research, statistical analyses and writing for the manuscript from this thesis (Chapter 2). My supervisors Andrew Trites and Trish Schulte provided input on experimental design, data analysis and editing. Dom Tollit acquired funding for the project, undertook Feeding Experiment 1 and provided guidance in the early stages of the project. Bruce Deagle provided assistance throughout my study.

Chapter 1 General introduction

Knowing what and how much animals consume is fundamental to wildlife management. This is particularly true for marine mammals that are commonly thought to compete with fisheries and contribute to reductions in fish stocks (Trites, 2003). However, obtaining this information can be difficult because marine mammals often forage below water and out of sight. This has resulted in the development of many indirect dietary analysis methods. These methods include identifying prey hard remains (otherwise known as hard parts) which are recovered from faeces (Tollit et al., 2006; Winship et al., 2006; Trites, Calkins and Winship, 2007), decoding fatty acid signatures in blubber (Iverson et al., 2004), quantifying stable isotope signatures in many different tissues (Hobson et al., 1996) and more recently identifying prey DNA from faeces (Purcell et al., 2004; Deagle et al., 2005; Tollit et al., 2006). The importance of knowing what and how much marine predators eat is emphasized by the extensive effort that has gone into developing these multiple methods of diet analysis.

Current methods of diet analysis in pinnipeds

There are currently four main methods for analyzing the diet of seals and sea lions (pinnipeds). The earliest methods used hard parts found in stomachs and faecal samples. These were followed by the development of two biochemical methods to determine diet from prey fatty acids and stable isotopes that have been deposited in predator tissues. The most recent technique uses prey DNA signatures from stomach and faecal samples of predators.

Each of these techniques was developed to provide an alternative way to interpret dietary information or to address shortcomings and biases with the methods that predated them.

Conventional hard part analysis

Enumeration of hard parts found in stomachs and more recently faecal samples has been the most common way to determine pinniped diets (Trites et al., 2007). Early diet analysis relied on observations of prey in the stomachs of animals caught in fishing nets or intentionally shot for dietary analysis (Pitcher, 1980). However, lethal sampling is not ethically acceptable in most cases. Techniques were experimented with to extract stomach contents without killing animals, but these remain problematic (Pitcher, 1980; Harvey and Antonelis, 1994; van den Hoff, Burton and Davies, 2003). Pinniped diet analysis moved to collecting faecal samples in the 1970s using primarily otoliths (an ear bone) from prey species which survived digestion and could often be identified to the species level (Frost and Lowry, 1980; Tollit and Thompson, 1996).

Controlled feeding studies illuminated considerable biases in prey representation using only otoliths for prey identification, which led to the use of other hard parts from prey (such as vertebrae) to provide a more complete picture of the diet (Pierce and Boyle, 1991; Tollit et al., 2003). Prey bones from a given meal are passed through the digestive system over 1-3 days, indicating that the hard parts found in a scat should reflect a spectrum of recent foraging rather than individual meals (Tollit et al., 2003). There are various ways to enumerate the hard parts in diet, including frequency of occurrence, percentage by number, and biomass reconstruction (Hyslop, 1980; Laake et al., 2002; Tollit et al., 2003; Tollit et al., 2007). Unfortunately, each of these methods provides different composition estimates.

Furthermore, including additional structures for prey identification does not reduce problems with differential digestion of hard parts and enumerating prey that have fragile or few hard parts.

Correction factors have been developed to account for differences in digestion of some prey species, but not all biases can be corrected (Tollit et al., 2003; Tollit et al., 2007). Factors that continue to affect the accuracy of diet reconstruction using hard-part analysis include the activity level of the predator, an inability to see prey species that have been regurgitated, or for which only part of the prey has been consumed (i.e., bellies of salmon), and the lack of hard parts in some prey. It is also interesting to note that the rate of digestion of prey bones seems to be predator specific, making it necessary to develop correction factors for every predator-prey system (Tollit et al., 2007). In addition, there is usually no way to identify the defecator using hard parts, and therefore no way to make dietary comparisons within a group of predators or between sexes. Despite these problems, hard part analysis can provide extremely useful information about the biomass or physical weight of prey consumed, which no other diet-analysis technique developed to date can do (Laake et al., 2002). In an attempt to overcome some of the limitations of hard part analysis, biochemical and DNA-based methods have recently been developed.

Biochemical methods

Fatty acid (FA) analysis detects prey species in predator diets by identifying FA in the predator's tissues that match those of prey species. Fatty acids can be used to provide information on predator diet because theoretically prey species have unique FA compositions or signatures, which are subsequently deposited into the predator's fat stores, creating a

profile of the prey species consumed, or a “fatty acid signature” (Colby, Mattacks and Pond, 1993; Iverson, Frost and Lowry, 1997; Hooker et al., 2001; Iverson et al., 2004). Fatty acid analysis is commonly used to 1) examine diet variation geographically or temporally or between groups of individuals, and 2) to obtain a proportion of prey species consumed by animals, termed quantitative fatty acid signature analysis (QFASA) (Iverson et al., 2004). Fatty acid analysis does not have the same problem with differences in digestion that hard parts does, and can be used to determine diet over the long term.

The strengths of FA analysis over other techniques are tempered by a number of potential caveats. These include variability in the fatty acid profiles of prey groups both geographically and temporally (Iverson et al., 1997), changes in the fatty acid profiles of prey after consumption by the predators (Iverson et al., 2004), differential deposition of the prey fatty acids by the predator (Hooker et al., 2001), and questionable taxonomic resolution (Bradshaw *et al.*, 2003; Iverson *et al.*, 2004). In addition, high-sensitivity to species-specific calibrations and false positives have been identified in some species such as harbour seals (*Phoca vitulina*) (Nordstrom et al., 2008). Such complicating factors require that extreme caution be used in applying fatty acid analysis to determine the diets of pinnipeds.

Like fatty acid dietary analysis, stable isotopes from prey species are deposited in predator tissues, providing a “signature” of the prey species consumed. This “signature” consists of relative ratios of the heavy and light isotopes of carbon and nitrogen (Hobson *et al.*, 1996; Hobson *et al.*, 1997). The heavy isotope of N is enriched as trophic level increases, and the ratio of the heavy to light isotope can be used to assess trophic position (Hobson et al., 1997). Carbon isotopes provide information on a horizontal scale, and can indicate if predators forage near the shore or further out to sea for example, or whether predators have

fed in fresh versus salt water. Different tissues retain isotope signatures for different lengths of time, providing different snapshots of diet, with some tissue providing information about diet over years (Hobson *et al.*, 1996; Sinisalo *et al.*, 2008). However, fatty acid analysis does not provide good taxonomic resolution (Fuller *et al.*, 2004; Fuller *et al.*, 2005), and stable isotope analysis is most useful for studying diet over a longer time scale, between sexes or for identifying trophic position or changes in foraging location. Cumulatively, fatty acid signature analysis and stable isotopes can provide useful information about trophic position and diet over the long term, but there are limitations to both methods, suggesting that a different method of diet analysis is necessary to account for the biases of hard part analysis.

DNA dietary analysis

DNA-based methods determine the prey in a predator's diet by amplifying prey DNA from DNA that has been isolated from the stomach contents of predators, or more commonly from the soft-matrix of faeces. Many molecular techniques and variations of techniques have been applied to dietary analysis, including monoclonal antibodies, restriction length polymorphisms (RFLP) and polymerase chain reaction (PCR) based methods (King *et al.*, 2008). Of these techniques, PCR-based methods are the most straightforward, easy and economical to apply (Jarman, Deagle and Gales, 2004; Deagle *et al.*, 2005; King *et al.*, 2008).

One of the significant challenges with PCR-based methods is the dominance of predator DNA in DNA isolated from faeces (Deagle *et al.*, 2005; Deagle and Tollit, 2007). To avoid complications resulting from the abundant predator DNA, predator DNA has to be stopped from amplifying, or primers need to be very prey specific so that amplification is not swamped by predator DNA. Group-specific PCR appears to be an efficient way to detect

prey species while simultaneously excluding predator DNA (Jarman et al., 2004; Deagle et al., 2005). Real-time PCR has also been shown to successfully amplify prey DNA without amplifying DNA from the predator (Deagle and Tollit, 2007). In comparison to hard-part analysis, studies have shown that DNA-based methods may be able to increase the number of species seen in a diet, improve taxonomic resolution and detect species that cannot be detected using hard parts (Tollit et al., 2009). However, although DNA-based analysis addresses some of the concerns with traditional dietary analysis, it continues to be a challenge to quantify the amount of prey species consumed by pinnipeds.

Recent studies have demonstrated that it may be possible to estimate quantities of prey consumed by pinnipeds using DNA. Monoclonal antibodies, real-time PCR and next generation sequencing have all been used to produce an estimate of the proportion of prey species consumed. Two studies applied clone libraries (Deagle et al., 2005) and real-time PCR (Deagle and Tollit, 2007) to DNA from faeces from Steller sea lions (*Eumetopias jubatus*) fed known amounts of known prey, and showed potential differences in digestion of prey DNA or different DNA densities in prey species. Further work has not been done to investigate clone libraries as a dietary analysis technique possibly because it is time-intensive, limiting the number of samples that can be analyzed. In their 2007 study, Deagle and Tollit developed correction factors for DNA density, and indicated that it may be possible to quantify diet within approximately 11% using real-time PCR. However, these results were based on a single diet consumed by captive sea lions, and therefore it was not clear if this method could be applied to other prey or predator species. Real-time PCR is promising as a method for diet quantification, but more extensive validation is necessary.

Recently, next-generation sequencing was applied to faecal DNA from wild Australian fur seals (*Arctocephalus pusillus doriferus*) to determine the proportions of prey consumed (Deagle, Kirkwood and Jarman, 2009). Deagle et al (2009) found that it was possible to determine a quantity consumed for prey species that were not consistently found using hard parts, and that this technique could be useful to quickly determine the diet of a large number of animals. However, this technique is currently expensive and has not been tested using controlled feeding studies. Further investigation is needed using captive animals that can be fed known diets to determine if the quantity of prey consumed can be accurately determined.

Diets of Steller sea lions

Developing an effective method of diet reconstruction is particularly important for Steller sea lions because of the drastic declines of their population in the Gulf of Alaska and Aleutian Islands (Trites and Larkin, 1996; Winship and Trites, 2006). Steller sea lions range from northern California to British Columbia and Alaska, and across the Aleutian Island to Russia and northern Japan, with the largest concentration residing in western Alaska (Aleutian Islands and Gulf of Alaska) (Loughlin, Perlov and Vladimirov, 1992). The western population of Steller sea lions has declined since the late 1970s, while the eastern population has increased slowly (3% per year, SE Alaska to California). The cornerstone of many of the hypotheses to explain these population declines is nutritional stress, or a reduction in the quality or quantity of prey species in the diet (DeMaster and Atkinson, 2002; Trites and Donnelly, 2003; Winship and Trites, 2003; Rosen and Trites, 2005; Nordstrom et al., 2008).

Steller sea lions are known to consume primarily small schooling species of fish and invertebrates. Studies have shown that these prey species include herring (*Clupea palasii*), walleye pollock (*Theragra chalcogramma*), Atka mackerel (*Pleurogrammus monopterygius*), capelin (*Mallotus villosus*), squid (Order Teuthida), salmon (Family Salmonidae), Irish lords (*Hemilepidotus spp.*), arrowtooth flounder (*Atheresthes stomias*), rockfish (*Sebastes spp.*) and a host of others (Sinclair and Zeppelin, 2002; Trites et al., 2007). Hard part analysis has shown that the western population of animals switched from consuming a large proportion (79%) of high fat fish (i.e., herring, salmon) and a small amount (32%) of lower fat gadids (i.e., pollock) in the late 1970s to almost the reciprocal in the 1990s – 85% gadids and 37% higher fat fish (Merrick and Loughlin, 1997). The regions with the lowest diet diversity declined the most (Merrick and Loughlin, 1997; Winship and Trites, 2003; Trites et al., 2007). Since the western population has declined, pollock have been the most common species consumed by sea lions in the Gulf of Alaska, and Atka mackerel have been the most common off of the Aleutian Islands, while the diet of animals in the increasing populations further south is considerably richer and dominated by fattier fish like herring and salmon (Trites et al., 2007).

Better diet quantification methods will contribute significantly to understanding the relationship between diet and population change. Estimates of diet composition in the western and eastern populations of sea lions have been derived from hard parts from faeces. However, since some prey cannot be identified to species level from hard parts (particularly salmon and rockfish species) important prey species that do not have hard parts may be missed (Tollit et al., 2009). In addition, the methods used to determine diet composition estimates for the western and eastern populations of these animals have not been consistent.

Both frequency of occurrence and biomass reconstruction methods have been used, and are known to produce considerably different estimates (Tollit et al., 2007).

My thesis research was designed to improve dietary analysis by developing and validating a method that will allow the proportion of prey consumed to be calculated. This will enable comparisons to be made between the diet composition of regional groups of Steller sea lions, possibly revealing major differences in diet diversity or predominance of one prey type relative to another.

Thesis theme and objectives

The holy grail for dietary analysis is to be able to determine the quantity of prey species that are present, not only the proportion, but also the biomass or weight of prey species consumed. Currently, diets of Steller sea lions are enumerated using prey hard parts recovered from their faeces, but this method has many inherent biases that may preclude accurate identification and quantification of prey. For example, squid have few hard parts (beak, eyeballs and a stratolith), and the beaks have been shown to get caught in sea lion stomach linings and digestive tracts, whereas some fish have extremely robust hard parts that pass easily through the digestive tract. Thus, determining the relative contributions of cephalopods and fish to Steller sea lion diets is a challenge using traditional diet analysis methods (Bowen, 2000).

The soft tissue of prey species is passed through the digestive system more consistently than hard parts (Deagle et al., 2005) and thus should not suffer from the same problems as dietary analysis using hard parts. Also, many recent studies have shown that DNA-based methods can increase the number of prey detected, and be more species specific

(Casper *et al.*, 2007; Tollit *et al.*, 2009). However, studies using more recently developed DNA-based methods have shown that quantitative estimates in diet may be precluded by differences in DNA density or digestion of prey species (Deagle *et al.*, 2005; Deagle and Tollit, 2007).

The overall objective of my thesis was to determine if the quantify of specific prey items consumed by Steller sea lions can be determined from the DNA in their faeces using real-time PCR, and whether there are certain diets for which proportional estimates are better calculated than others. The results of my research are presented in a manuscript format (Chapter 2). This results in some necessary repetition of material between the three chapters of my thesis. The research presented in Chapter 2 describes and validates a molecular technique that can quantify the diets of Steller sea lions in the wild. This technique overcomes many of the biases associated with traditional diet reconstruction methods, and may provide a starting point for quantitative diet analysis of other predator species.

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Chapter 2 ¹Quantity of prey consumed can be determined from faecal DNA using real-time PCR

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, reconstructing the diets of some species such as pinnipeds is challenging if foraging cannot be directly observed. Traditionally, the diets of pinnipeds have been described from the presence of prey species and hard parts retrieved from their stomachs (Scheffer, 1928; Frost and Lowry, 1980; Prime and Hammond, 1987), and have more recently been determined from the presence of prey bones and other hard parts recovered from faecal samples (Merrick and Loughlin, 1997; Sinclair and Zeppelin, 2002; Tollit et al., 2007; Trites, Calkins and Winship, 2007; Tollit et al., 2009). However, accurately resolving and quantifying the species consumed from faecal samples has been limited by differences in digestion among species and by the lack of hard parts in some prey (Bowen, 2000; Tollit et al., 2007; Tollit et al., 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 time-scales, but are unable to provide species-level resolution (Hobson et al., 1996; Hobson et al., 1997; Sinisalo et al., 2008). In contrast, quantifying the proportions of fatty acids of different prey species deposited in pinniped

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blubber has had mixed success due to inconsistencies in prey fatty acid signatures, high sensitivity to prey-specific calibrations, and biases associated with rates of assimilation of prey fatty acids (Tollit et al., 2007; Nordstrom et al., 2008; Meynier, 2009). DNA based techniques on the other hand have been more successful at identifying the presence or absence of specific species using faecal samples (Jarman, Deagle and Gales, 2004; Casper et al., 2007; Deagle and Tollit, 2007; Tollit et al., 2009). Their major advantage over fatty acid and stable isotope techniques is that they can determine diet on a finer time scale (i.e., days) and can identify the species of prey consumed with greater accuracy (Deagle et al., 2005; Deagle and Tollit, 2007; King et al., 2008; Tollit et al., 2009).

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 and Tollit, 2007; King et al., 2008). However, only a few studies to date have attempted to determine quantities of prey species consumed from the presence of DNA in faeces (Deagle et al., 2005; Ball et al., 2007; Deagle and Tollit, 2007; Matejusova et al., 2008; Deagle, Kirkwood and Jarman, 2009). Two of these studies tested whether the 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 and Tollit, 2007). These studies successfully identified the prey species fed, but could not accurately estimate the proportions of prey species consumed due to species-specific differences in DNA density or survival during digestion. However, applying correction factors for mitochondrial DNA (mtDNA) density of the prey resulted in consumption estimates that were within ~11% of the proportions fed (Deagle and Tollit, 2007). Such results are promising, but need further testing and development through additional feeding experiments using more prey species.

The goal of our study was to undertake a comprehensive validation of real-time PCR to estimate percentages of prey consumed using DNA extracted from faeces. We did so by first 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. Our study shows that relative amounts of prey consumed by Steller sea lions can be determined from the amounts of DNA present in their faeces, and that the technique can be readily applied to a wide range of apex predators.

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 COI for squid (*Loligo opalescens*) were designed using Primer Express Software (v2; Applied Biosystems Inc.). Note that some of the primers we used (Table 2.1) match closely to species that were not in our study based on comparisons to all available sequence data (using the NCBI BLAST tool), and are 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 homogenized soft tissue 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

Table 2.1. Real-time PCR primers used in this study.

<i>Accession number</i>	<i>Target gene</i>	<i>Species</i>	<i>F-forward or R-reverse</i>	<i>Sequence 5'-3'</i>
EU548092	16S	<i>Clupea palasii</i>	F	CGCCCACCAATCACGAA
			R	ACGTTTGTGCCAGTATCACGTT
EU548154	16S	<i>Thaleichthyes Pacificus</i>	F	GAGAAGACCCTATGGAGCTTTAGACA
			R	GGAGTCACAATGTTTTTTCCCTTT
AF000051	CO1	<i>Loligo opalescens</i>	F	TTAGCATCCTCGGCTGTTGA
			R	CCAGCATGAGAGAGATTCTAGATAGG
EU548166	16S	<i>Sebastes helvomaculatus</i>	F	GAGCACCCCTCCTACAATTA
			R	CGGCATTGCCGGATCTTA
EU548165	16S	<i>Sebastes alutus</i>	F	GAGCACCCCTCCTACAATA
			R	CGGCATTGCCGGATCTT

* F and R denote forward and reverse

al. (2005) and Deagle and Tollit (2007). The only exception was that we eluted DNA in the AE buffer provided with the kit. Mean amount of starting material was 93.6 ± 26.9 mg, and 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 1 cycle of 50° / 2 min, 95° / 10 min, 40 cycles of 95° / 15 s, 60° / 1 min, 1 cycle of 95° / 15 s, 60° / 20 s, 95° / 15 s. Reaction volume was 22 μ l, containing 4 μ mol of each primer and 2 μ l 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 μ l SYBR Green Master mix (Applied Biosystems Inc).

A 2-fold serial dilution (ranging from 4 ng total DNA in the well to 0.0325 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 for amplification. 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 differences in mitochondrial DNA content per gram total DNA in the tissue when known amounts of total DNA from each prey species are mixed prior to real-time PCR.

Estimates of 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 mixed the two species to the percentages specified above. The final concentration of each mix was also 2 ng/ul. We then used real-time PCR to estimate the total quantity and proportion of species DNA in the mix. In short, we obtained quantities for each prey species for each mix by real-time PCR, combined them to make a total quantity, and divided to get a proportion, which we then multiplied by 100 to obtain a relative percent contribution to each 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 2.2). We chose these four prey species because 1) herring is one of the staples of sea lion diet based on hard part analysis, 2) eulachon is eaten in the wild and is similar in tissue composition to herring, 3) squid and other cephalopods are not always passed consistently (Bowen, 2000; Tollit et al.,

2003; Tollit et al., 2006), and 4) rockfish is a bony fish like herring and eulachon, but has a much lower fat content.

Estimate of percent of diet consumed (by weight) from Steller sea lion faecal DNA

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. The faecal samples we used were collected in two separate feeding experiments: 1) from Oct 12/06 - Feb 7/07, called “Feeding Experiment 1”, and 2) from Feb 16/09 – Apr 28/09, called “Feeding Experiment 2”. Six female Steller sea lions participated in the two studies. They were captured as pups from northern Vancouver Island and studied at the Vancouver Aquarium between the ages of 3-6 years old. All research was conducted under the 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 three years old and two (F00ED, F00YA) were six 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 DNA mix validation described above). Food intake (± 0.01 kg) was controlled daily. Faeces were collected regularly over the course of our study and subsamples of each faeces 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 six-year-old Steller sea lions (F03AS and F03RO), until a minimum of five scats had been collected for each diet (Table 2.2). 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). The sea lions were housed together while faeces were collected in an enclosure containing a haulout and pool. Each of the diets was consumed for about seven days.

There was a three-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 vacuumed). Scat was collected opportunistically from the pools and haulouts and while training sessions were in progress over the next four 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 tissue 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 of the gut, and DNA from the predator itself. As a result, the standard curve method outlined above will not correct for differences in mitochondrial DNA density among the prey

Table 2.2. Percentage by weight of prey species fed to Steller sea lions in Feeding Experiment 2.

<i>Diet</i>	<i>Herring</i>	<i>Eulachon</i>	<i>Squid</i>	<i>Rockfish</i>
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

Items for DNA from faeces (because the relative contribution of prey DNA to the total DNA extracted is unknown). We therefore derived a correction factor to adjust the amounts of prey species consumed (by weight) for mitochondrial volume density.

We developed species-specific mtDNA correction factors by estimating difference in the absolute amount of mtDNA for a given amount of total DNA among the various prey species. To develop these correction factors, we used DNA isolated from the tissues of the four prey species we studied and 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 reactions, and then using this to calculate PCR efficiency and amounts of mtDNA for each of the four prey species (herring, eulachon, squid and rockfish) according to:

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

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

$$(3) \textit{Amount of mtDNA per total amount of DNA added} = \textit{amount of mtDNA in sample} / \textit{amount of total DNA added to sample}$$

We used our calculated correction factors to correct the percentage of prey fed to the animals (expected value by weight) for mitochondrial volume density. 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 analysis was performed using R (R Development Core Team, 2008). 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 logit transformed the proportions and regressed the expected proportions against the average proportions observed for each diet. We then performed a *t*-test to determine if the slope of the line was significantly different from one.

Results

Estimates of percent of DNA in mixtures of prey DNA

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. 2.1). 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 correction factor for differences in mtDNA density among the prey species, and 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.

Table 2.3. Real-time PCR estimates of prey species in 7 mixtures of herring and squid DNA. Data are mean \pm standard deviation of 10 replicates.

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

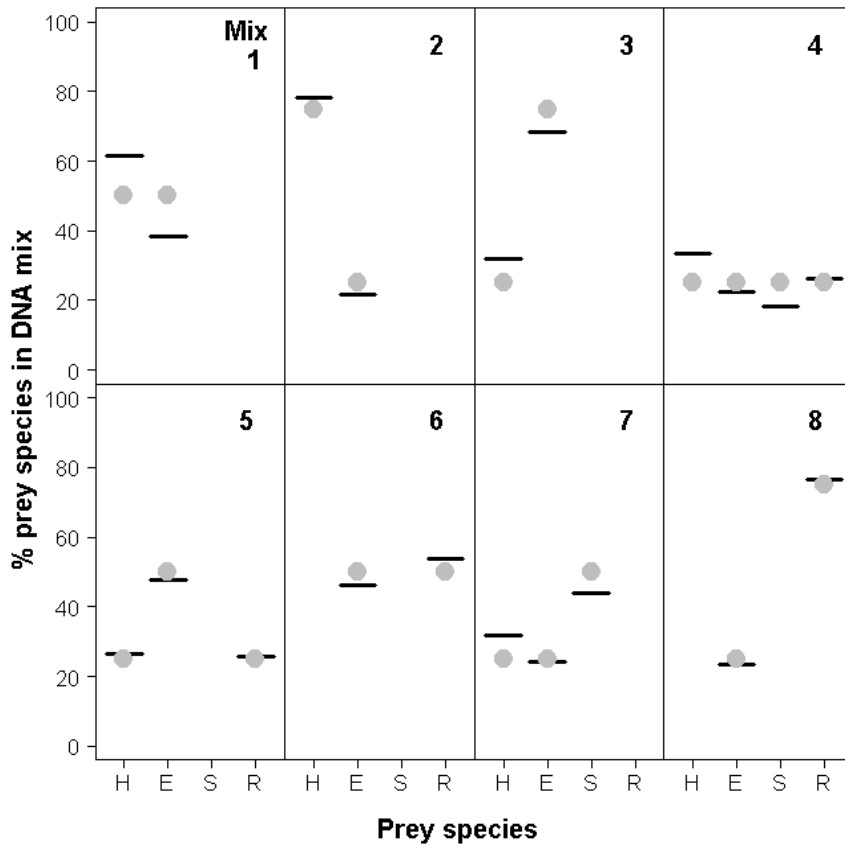


Figure 2-1. Real-time PCR estimates of prey species in 8 mixtures of prey DNA. Solid dots represent the percent of each prey species' DNA added to the mixture. Bars represent the percent of each prey species estimated to be present by real-time PCR. Prey species were H-herring, E-eulachon, S-squid and R-rockfish. Each mix was assayed in duplicate.

Estimates of percent of diet consumed (by weight and corrected for mtDNA density)

Feeding Experiment 1

Applying the above correction factors to the amount of mtDNA of each prey species recovered in faecal samples indicated that our study animals had eaten 87.7% herring, 9.9% eulachon, 1.99% squid, and 0.44% rockfish, when their actual diet had contained 64.3% herring, 14.3% eulachon, 14.3% squid, and 7.1% rockfish (by weight). As shown in Fig. 2.2, our estimated percentages of prey consumed were within ~17% of the expected values.

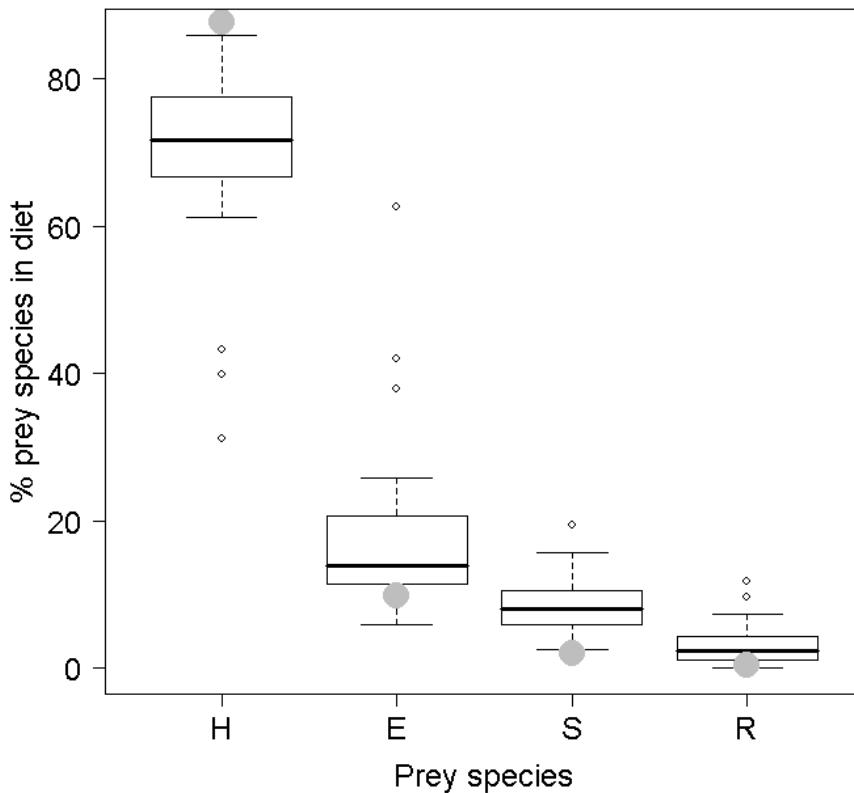


Figure 2-2. Real-time PCR estimates of prey DNA in Steller sea lion faeces from a diet of known composition. Solid dots represent the percentage of prey that was fed to the animals (by weight and corrected for mtDNA density). Boxplots show the median, range and upper/lower quartiles of the percentage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

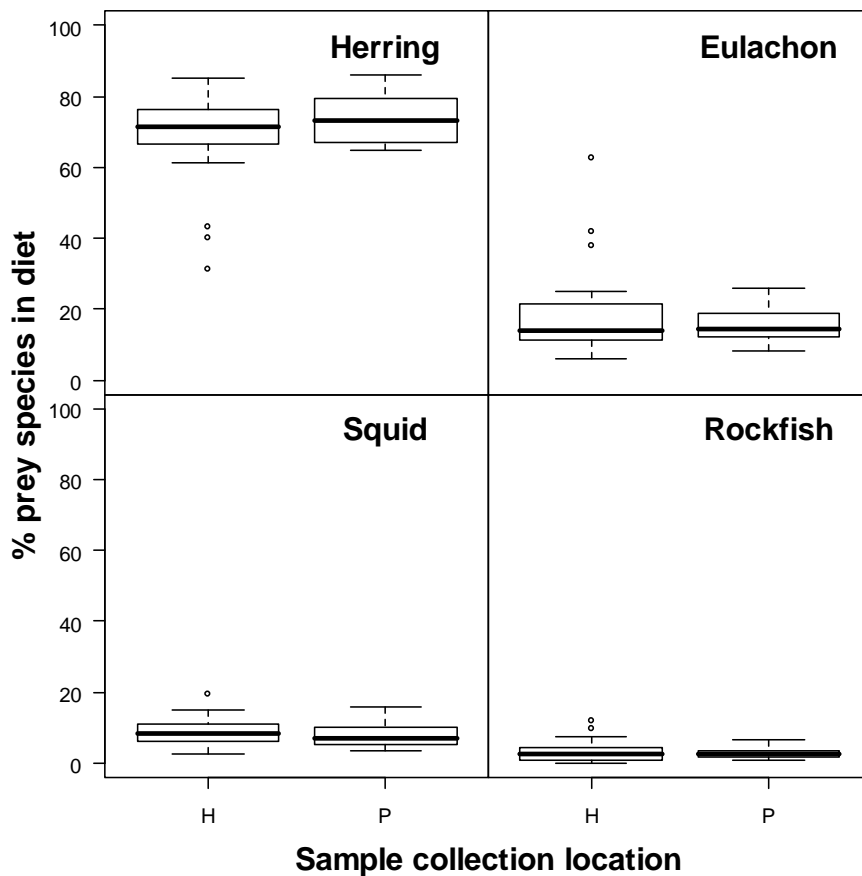


Figure 2-3. Real-time PCR estimates of prey in Steller sea lion diets for scats collected from the H-haulout or P-pool of the animals' enclosure. Boxplots show the median, range and upper/lower quartiles of the percentage estimated by real time PCR for samples collected from the haulout or pool. N = 36 from the haulout and 9 from the pool.

Average percentages of prey species in the diet did not differ appreciably when samples were collected from the pool or the haulout (Fig. 2.3). We had expected that faecal samples collected from the pool may not be representative of all the prey species that were present because faecal samples are not necessarily homogeneous masses (Deagle et al., 2005) and some prey species may wash away in water. Figure 2.3 illustrates that irrespective of sampling location, the estimated proportions are very close.

Feeding Experiment 2

The number of scats assayed for each of the ten diets containing herring, eulachon, squid and rockfish (Table 2.2) was 4 scats for Diets 2 and 4, 5 scats for Diets 3, 5, 7-10, and 6 scats for Diets 1 and 6. The accuracy of percentage estimates were similar to what was seen in both the mixes of DNA from tissue (Table 2.2 and Fig. 2.1) and Feeding Experiment 1 (Fig. 2.2) when the percentages of prey species expected by real-time PCR were corrected for mtDNA density. Overall, 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. 2.4).

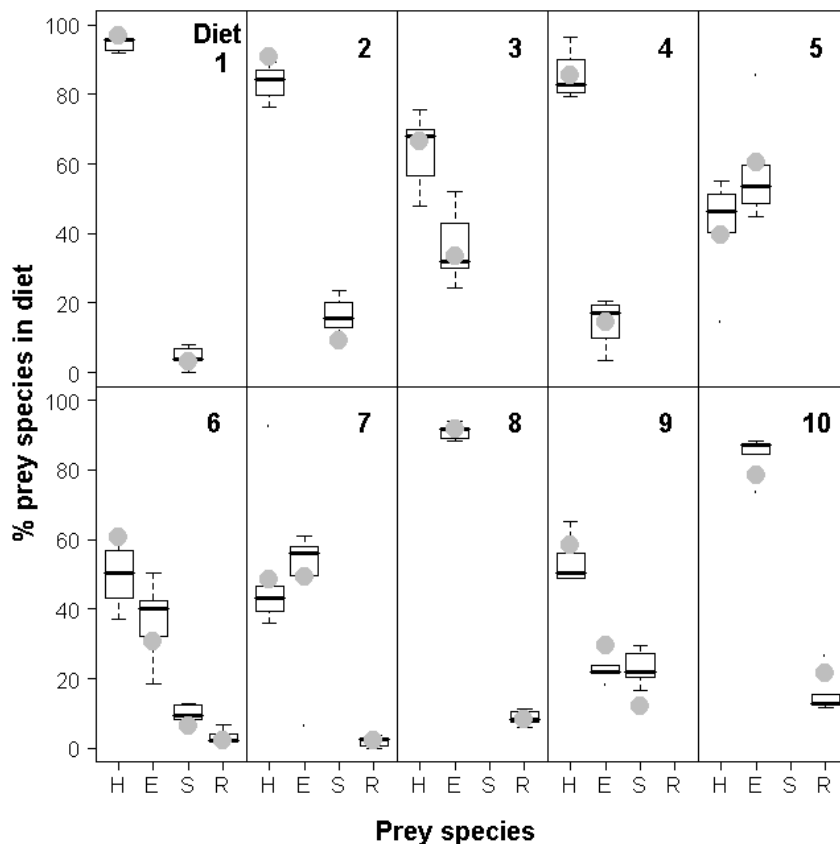


Figure 2-4. Real-time PCR estimates of prey DNA in Steller sea lion faeces from ten diets of known composition. Solid dots represent the percentage of prey that was fed to the animals (by weight and corrected for mtDNA density). Boxplots show the median, range and upper/lower quartiles of the percentage 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).

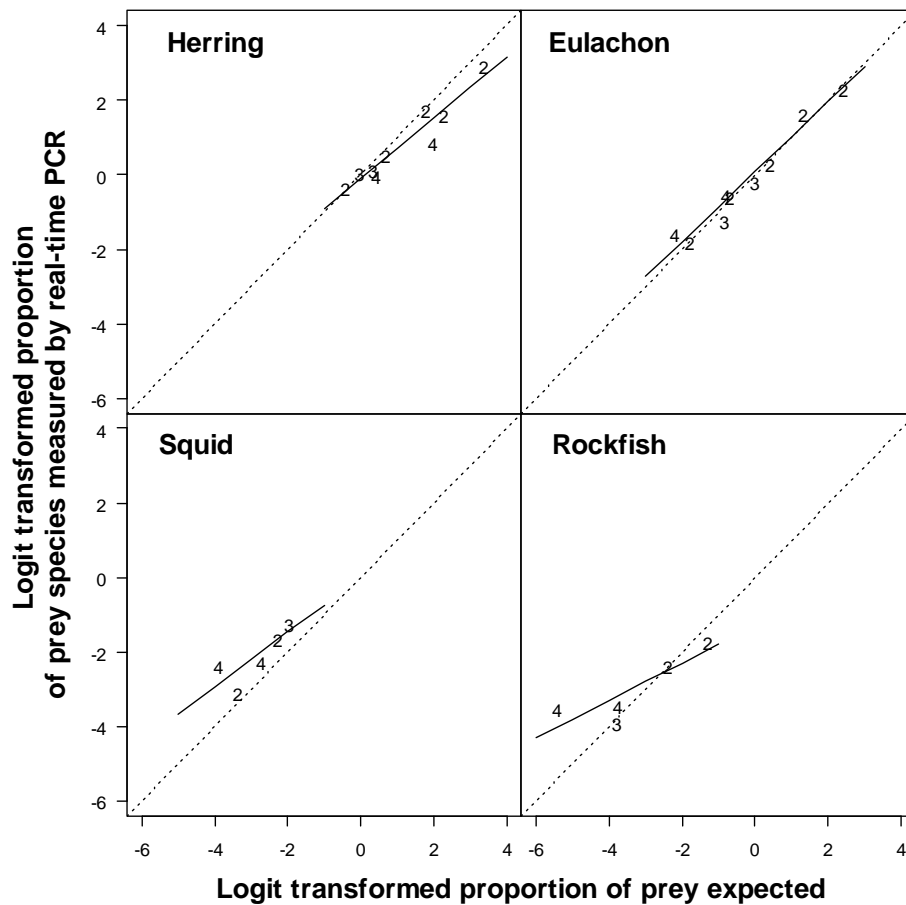


Figure 2-5. Proportion of prey expected and measured by real-time PCR in Steller sea lion faeces from eleven diets of known composition. Each diet is represented by a number, which shows the number of prey species that were tested in that diet. Both the proportions expected (weight fed and corrected for mtDNA density) and measured are logit transformed. The solid line represents the best fit line and the dotted line represents a slope of 1. N = 4 – 45 (see text for details).

The proportion of prey species measured by real-time PCR was not significantly different from that expected for any of the species we tested (two-tailed t -test, $\alpha = 0.025$; herring $p = 0.04$, eulachon $p = 0.22$, squid $p = 0.23$ and rockfish $p = 0.03$; N = 4 – 45; Fig. 2.5). It is evident that the regression for rockfish is being driven by one outlier (Fig. 2.5), making it appear that the proportion of DNA in the faeces does not exactly reflect the proportion consumed. Despite this, our results were robust over a wide range of diets, and the estimated percentages were always within 12% of the expected amount (which was the same

as the theoretical accuracy of the technique based on estimates from mixes of known amounts of DNA), except for herring in Feeding Experiment 1 which was within 17% of the expected amount.

Discussion

Real-time PCR can be used to determine the relative amount of prey consumed by Steller sea lions from the DNA contained in their faeces. We estimated the proportion of DNA contained in mixtures of prey DNA within 12% of the amount of DNA in the mix, and 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. This technique can be easily applied to samples collected from the field, and has many advantages to traditional diet analysis techniques.

Real-time PCR diet quantification

Deagle and 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 one tissue mixture homogenate containing herring, salmon and smelt, and later from the faecal DNA of sea lions fed the same combination of fish. However, unlike us, 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) as their standard curve for real-time PCR and 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). They therefore back-calculated correction factors for mtDNA density to obtain percentages of prey that fell within ~11% of

the prey fed. 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 and Tollit (2007) by completing a much more extensive validation of the technique. We fed eleven different diets of four species, in contrast to the previous work which examined only a single diet combination consisting of three species. Similar to Deagle and Tollit (2007) we found that mitochondrial DNA (mtDNA) density derived correction factors allowed fairly accurate estimates of diet proportions (12-17%). The mtDNA density derived correction factors were quite similar to correction factors derived empirically from the data obtained in the feeding trials. Using data from the diet in which the four prey species were fed in equal parts (25% for each of herring, eulachon, squid and rockfish), we estimated that the amount of DNA from each species represented in the faeces and expressed relative to herring was: 1.000 for herring, 0.746 for eulachon, 0.193 for squid and 0.063 for rockfish (compared with mtDNA density derived correction factors of 1.000 for herring, 0.506 for eulachon, 0.102 for squid and 0.046 for rockfish). Using empirically derived correction factors did not markedly improve the accuracy of our estimate of diet proportions, except in Feeding Experiment 1 where the difference between what was expected and what was obtained was reduced from 17% to 12% for herring. These data demonstrate that applying mitochondrial correction factors can produce similar accuracy to deriving correction factors from controlled feeding studies, and suggest that controlled feeding studies are not necessary for applying this technique to other prey species.

We also provided a more comprehensive validation of the theoretical accuracy of real-time PCR by analyzing mixtures of isolated DNA from four prey species in 15

combinations. These validations indicated that the theoretical accuracy of the technique was $\pm 12\%$, which suggests that our protocol performed well on faecal DNA with an accuracy of 12-17%. Developing a standard curve based on dilutions of genomic DNA instead of a plasmid (as in Deagle and Tollit, 2007) should also make future applications of this technique more accessible and practical. The extensive validations we undertook using many different mixes of DNA and diets fed suggest that real-time PCR is a very 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 once differences in mtDNA density have been accounted for. Correction factors based on mitochondrial density will be much easier and more practical to derive compared to performing feeding trials on captive animals for all potential prey species.

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 hard parts are that it is faster, more people are able to do it, and it is more repeatable. There are molecular labs readily equipped for real-time PCR at most universities and government institutes, and many molecular biologists that 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 are to some extent subjective and expensive relative to molecular methods, and require the application of correction factors (as in molecular analysis) to reconstruct the biomass of prey consumed. Calculating correction factors in hard part analysis, however, requires controlled feedings to correct for bone digestion and passage times of hard parts.

Thus, cumulatively quantitative molecular analysis may be cheaper, easier and more efficient than using hard remains to quantify diet.

Our data showed that all of the prey species that the Steller sea lions consumed over our feeding experiments were present in the soft-matrix of their faeces, unlike hard parts that are sometimes regurgitated or digested beyond the point of recognition. Real-time PCR is therefore not likely to miss prey species due to regurgitation or preferential consumption of parts of prey that do not contain diagnostic hard parts (e.g., salmon or cod bellies) (Tollit et al., 2003; Trites et al., 2007; Tollit et al., 2009). In addition, PCR based techniques are more species and group-specific, and can therefore quantify species of salmon and rockfish that cannot be identified from hard remains. DNA analysis can also detect species of prey that are rare and consumed in such low amounts that they are unlikely to have hard parts that survive the digestion process. Hard part diet reconstruction typically does not report prey types that are in < 5% of scats because the resolution of hard part analysis is insufficient to be confident of their importance in the diet (Trites and Joy, 2005). In our case, 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.

Although real-time PCR and DNA analysis can more accurately identify species of prey (Tollit et al., 2009) and the proportions consumed compared to hard-part analysis, they cannot determine the sizes eaten. 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 the ultimate 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, 9.9% squid, 1.99% 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 some *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 non-quantitative survey of many potential prey items in diet samples. For example, Tollit et al. (2009) used a group-specific PCR method whereby closely-related groups are amplified using PCR while simultaneously excluding the predator's DNA from amplification. 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 all 100+ species previously reported to be part of the Steller sea lion diet (Sinclair and Zeppelin, 2002; Trites et al., 2007), but this would be quite laborious and inefficient. 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

Determining the proportions of prey consumed by pinnipeds in the wild can be done from scats collected in the field using real-time PCR following five steps. First, all prey species that are in each scat sample need to be identified using a presence-absence PCR technique such as DGGE (described above). Based on computer models simulating the hard part frequency of occurrence method, a minimum of 59 scats should be sufficient to identify presence and absence of major prey groups for a predator, or a minimum of 94 scats if temporal or spatial comparisons of diet are required (Trites and Joy, 2005). 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 developed for mtDNA density using DNA extracted from tissue for the prey species of interest. Finally, the primers and correction factors designed can be used to analyze field samples according to our protocol.

Identifying secondary prey items as part of a predator's diet is a potential concern for all diet studies, but does not appear to be a problem for real-time PCR diet analysis. We

added 4 ng of DNA to each PCR reaction, 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 the average amount of prey DNA (for all four species combined, $n = 45$) was 0.0178ng — not even 0.5% of the total DNA added. This should be consistent between real-time PCR reactions because reactions were always between 90-110% efficiency. Given the physical amount of primary prey relative to secondary prey, the amount of prey DNA that is amplified from a faecal sample is sufficiently small that any secondary prey that is present is unlikely to amplify.

The molecular protocol that we developed should be applicable to any predator prey system. 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 (Deagle et al., 2005; King et al., 2008; Nejstgaard et al., 2008). In some cases, proposed techniques have not been validated in a controlled feeding environment to determine whether or not they misrepresent certain prey due to differences in mtDNA density or digestion (Deagle et al., 2009). Cumulatively, these studies show that it should be possible to implement our real-time PCR protocol with little additional validation to quantify prey species in other systems where processes of digestion are similar to Steller sea lions.

Conclusions

Overall, we demonstrated that real-time PCR is an effective way to quantify prey consumed by Steller sea lions. Correction factors for mtDNA density in prey species can be easily derived and applied to estimate the proportions of prey consumed. This molecular

technique works for a wide range of prey types with different mitochondrial DNA densities, and should also be readily applicable to quantify the diets of other predator-prey systems.

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Chapter 3 General conclusions and future directions

My thesis research examined whether or not it was possible to accurately estimate the quantity of prey consumed by Steller sea lions with real-time PCR using DNA recovered from their faeces. Answering this question was a two-step process (Chapter 2). I first examined whether or not real-time PCR could accurately estimate the proportions (expressed as percentages) of four undigested prey species (herring, eulachon, squid and rockfish) in a comprehensive set of mixtures of prey DNA. I then applied the technique to DNA from the faeces of Steller sea lions fed 11 diets of differing quantities of the same four prey species. My greatest concern was whether differences in digestion between prey species would prevent accurate quantification as encountered by Deagle and Tollit (2007) and Deagle et al (2005).

Summary of findings

I found that real-time PCR can accurately estimate the proportions of prey species in mixes of prey DNA, as well as from the DNA from the faeces of Steller sea lions (after correcting for the relative mitochondrial DNA densities of prey species). Applying real-time PCR to the mixes of prey tissue DNA accurately estimated composition within ~12% of the amount of DNA in the mix. Using these mixes of DNA, I found that herring, eulachon, squid and rockfish had very different amounts of mtDNA (mitochondrial volume densities). Real-time PCR was further able to determine the proportion of all prey species fed to Steller sea lions in eleven different diets within approximately 12 - 17% of the amount fed. However, this required correcting the amounts of prey fed (by weight) for the relative mtDNA content

in the prey species. I also found that whether a sample was collected from the water or on land did not affect my ability to predict the proportion of prey species that are in a diet. My study demonstrates that real-time PCR works for different diets and prey species that have different mitochondrial volume densities.

Strengths and weaknesses

I believe the most significant contribution of my study was validating real-time PCR as a technique for determining diet composition from faecal samples. I analyzed 15 different DNA mixes with DNA from prey species ranging from 2-15% contributions to a diet, and with absolute amounts of DNA that were similar to what I would expect from DNA extracted from faeces. I also analyzed faecal samples from sea lions fed 11 combinations of prey species that ranged from 7-75% contributions to their diet (by weight). The prey I tested included a fish and squid species that are often misrepresented in diet analysis using hard-parts (Tollit et al., 2003). My extensive validations provided confidence that quantitative diet estimates using real-time PCR will not be biased by differences in digestion of prey species, or by diets that consist of predominantly one prey type or many minor contributors.

Another important strength of my study was its simplicity. I used genomic DNA isolated directly from the prey species for my standard curves as opposed to a plasmid (used by Deagle and Tollit (2007)). Plasmids are made by inserting sequences of the prey species to be amplified from faeces into bacterial DNA to make a strand of DNA that contains sequences from all prey species. Standard curves by either method are serial dilutions of DNA that are used in real-time PCR to calculate the amount of DNA in a sample of unknown quantity (i.e., the quantity of a prey species in a faecal sample). However, it is far more

labour-intensive to produce standard curves from plasmids. In addition, I demonstrated that correction factors can be easily developed using mixtures of the isolated prey DNA, and that it is not necessary to complete controlled feeding studies for this purpose. The simplicity of calculating correction factors from mixtures of prey DNA and using genomic DNA for standard curves should minimize the effort needed to apply this technique to other Steller sea lion prey species and prey in other systems.

The most significant drawback to my study is that I did not apply real-time PCR to samples from wild Steller sea lions or from other systems. In principal, the two-step “group-specific to real-time PCR” protocol that I proposed in Chapter 2 should work when applied to field samples, but I do not know if there will be intricacies or complicating factors. In addition, there may be aspects of digestion that might render real-time PCR ineffective for other predators. Applying real-time PCR using my protocol to samples from wild Steller sea lions and other predator prey systems is a logical next step.

Implications and future research

My results show that real-time PCR should be an accurate, easy and efficient way to analyze the diets of Steller sea lions in the wild, and should be applicable to other predator prey systems. Since real-time PCR relies on DNA from the soft matrix of faeces instead of hard parts, diet estimates are not affected by differential digestion of hard parts, or by the lack of hard parts in some prey. My protocol should also be applicable to any prey species because it depends on a database of sequences that is easily accessible from anywhere in the world instead of depending on a rare reference library of prey hard parts. Also, it is relatively easy and inexpensive to obtain any sequences that are not in the database. Hard part analysis

is further restricted because correction factors developed for prey bones from one species cannot be used for the same prey that have been eaten by a different predator (Tollit et al., 2007). This is not the case for DNA. Real-time PCR estimates of the proportions of prey consumed can be incorporated into bioenergetic models to predict the amounts of different types of prey that are required by sea lions (Winship, Trites and Rosen, 2002; Winship and Trites, 2003). Better estimates of diet composition will improve understanding of regional differences in diet, and will assist fisheries managers in making decisions about the resources caught by people and consumed by sea lions.

It will be important to determine the number of faecal samples that are required to have confidence in spatial, temporal or within-group comparisons of the proportion of prey species in diets. In Chapter 2, I indicated the number of faecal samples that should be analyzed to detect the presence or absence of prey species in a diet, but this is based on computer models simulating the hard part frequency of occurrence technique (Trites and Joy, 2005). Frequency of occurrence estimates are based on presence absence data, whereas my method determines a proportion of prey species directly. These differences lead to different model assumptions. Computer simulation modeling should be used to determine the number of scats that are necessary to make a wide range of population level comparisons using real-time PCR.

The accuracy of diet reconstruction using real-time PCR should not be affected by whether the scat was collected from water or on land if prey are passed in meal-specific pulses. Our data showed that the location of sample collection did not affect the estimated proportions of prey species consumed. However, the samples that we used for this analysis (36 scats from land and 9 from water) were from a single diet experiment for which the same

diet was fed for a long period of time, and it may be important to look more closely at passage rates of prey species over many different diets.

Real-time PCR could be made more efficient for dietary analysis by developing a multiplex reaction where many prey species are quantified simultaneously. In my study, I amplified only one prey species at a time and therefore had to run samples separately with primers for each prey species. However, many PCR-based studies use multiple primer sets per reaction (Bowles et al., 2007; Harper et al., 2005; Molenkamp et al., 2007). In these experiments, primer sets are designed so that the products have different lengths, and can therefore be easily differentiated. This should reduce analysis time greatly, thereby increasing the number of samples that can be processed in a given period. This could be important for conservation purposes when large numbers of samples need to be processed quickly.

My study demonstrates that the proportion of prey consumed can be determined with relative accuracy, but that the size (length and weight) of the species consumed cannot be determined from molecular techniques. Proportional estimates using DNA based methods are not inhibited by the effects of digestion, regurgitation and lack of hard parts. However, the only way to estimate prey size is from equations that relate body size to the lengths of hard parts recovered in scats such as otoliths and beaks (Laake et al., 2002; Tollit et al., 2007). Therefore, it would be useful to develop a model that can combine information about the weight of prey consumed using hard parts and that of molecular analysis to provide a more complete picture of the diet.

Next-generation sequencing such as pyrosequencing (also known as 454) has recently been proposed as a method for quantifying predator diets. Deagle et al (2009) combined

group-specific PCR with pyrosequencing to determine the relative proportions of prey consumed by Australian fur seals. They were able to determine the relative proportion of both individual prey species and higher-level groups for a large number of samples to assess population-level trends in diet. In fact, they also detected species that were difficult to assess using hard parts. However, next generation sequencing techniques have not been tested in a controlled feeding environment. Since all previous studies applying quantitative techniques to analyze diet have shown that differences in prey DNA densities need to be accounted for, controlled feeding studies should be applied to assess this technique more thoroughly. In addition, pyrosequencing is much more expensive than real-time PCR, and it will likely be some years before the price is reduced enough to make it accessible to many groups that are looking to complete population level analyses of diet. It would be useful to compare results and cost of real-time PCR and pyrosequencing to assess the long-term use of these techniques.

My study confirmed that real-time can be used as a dietary analysis technique in Steller sea lions. The next step is to apply the protocol outlined in Chapter 2 to samples from wild animals. As is evidenced by the recent pyrosequencing study by Deagle et al. (2009) and other real-time PCR studies (Matejusova et al., 2008; Nejstgaard et al., 2008), quantitative diet analysis is in demand, and new techniques will continue to emerge to accomplish this. These techniques will contribute to the conservation of pinnipeds and other marine species by improving our knowledge of their nutritional needs. Real-time PCR analysis of DNA recovered from faecal samples is a significant stepping stone to improving dietary analysis.

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