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DNA-based identification of salmonid prey species in seal faeces

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Abstract

Assessment of pinniped predation most often relies on analysis of the hard, undigested prey remains evident in faecal (scat) samples. For many prey species this method can yield valuable information on predator–prey interactions. For some genera, however, species diagnostic characteristics are lost during the process of prey digestion, thereby preventing morphological identification of fish prey species. Here, the feasibility of using faecal DNA to detect the presence of salmonids in pinniped scat samples and to distinguish reliably between sea trout *Salmo trutta* and Atlantic salmon *S. salar* was assessed. Novel salmonid mitochondrial DNA (mtDNA) primers were designed to amplify 162 bp of the 16S rDNA and a 327 bp section of the cytochrome *b* gene. Species-specific banding patterns were obtained by digestion of the cytochrome *b* PCR product with the restriction endonuclease *Alu*I, and confirmed by the species-specific amplification of the 16S rDNA fragment from Atlantic salmon. Scats collected from captive grey seals *Halichoerus grypus* fed on known monospecific diets used to validate the PCR-RFLP assay indicated a probability of at least 95.8% (23 of 24 faecal extracts) of detecting salmonids using DNA extracted from the scat matrix. Implemented alongside conventional prey remains analyses, this technique presents a promising new method for examining prey composition and assessing pinniped predation on salmonids.

Key words: pinniped, marine mammal, faeces, cytochrome *b*, prey identification

INTRODUCTION

A reliable estimate of diet composition is the foundation for understanding the trophic effects of pinniped predation. To date, much of the focus has been on the interaction between pinnipeds and commercially important prey species owing to direct competition for a limited resource (Harwood & Croxall, 1988; Yodzis, 2001). Such interactions are heightened when the prey species in question are valuable, and/or endangered, and as such there are particular concerns about the impacts of seal predation on stocks of both Atlantic and Pacific salmonids (e.g. Middlemas, 2003; Middlemas, Armstrong & Thompson, 2003; Orr *et al*., 2004; Purcell *et al*., 2004).

Traditionally, prey identification has relied upon morphological identification of the undigested hard remains (e.g. cephalopod beaks, fish sagittae, and other bones) contained either within the digestive tract or in collected scat samples (Pierce & Boyle, 1991; Tollit *et al*., 1997). Although the analysis of hard parts has made significant contributions to the study of pinnipeds and their feeding habits (e.g. Harkonen, 1987; Prime & Hammond, 1990; Pierce & Boyle, 1991; Hammond, Hall & Prime, 1994), potential biases from the application of these methods have been pointed out (Jobling & Breiby, 1986; Tollit *et al*., 1997). In particular, biases will occur unless species-specific differences in (1) the reduction in hard part size as a result of partial digestion (Prime & Hammond, 1987; Harvey, 1989; Tollit *et al*., 1997), and (2) the proportion of otoliths that are completely digested (e.g. Bowen, 2000) are taken into account.

Although analyses of hard prey remains in pinniped scats that incorporate these correction factors may work adequately for many species of prey fish, there are additional problems specific to the identification of salmonid species. Only a small proportion of salmonid otoliths may be represented in scat samples because they are more fragile than otoliths of other prey species (Boyle, Pierce & Diack, 1990). In addition, it has been suggested that seals may not consume the heads or skeletal elements of large prey (Pitcher, 1980; Orr *et al*., 2004). Although there is no evidence for this for fish taken in the open sea,

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it may be a significant problem for fish taken from static nets. This potentially low intake, coupled with high loss rates, could result in an inability to obtain representative results even with the application of correction factors. Additionally, assignment of salmonid species (particularly distinction between salmon and sea trout) based on otoliths is confounded by the loss of distinguishing characteristics during digestion processes (Middlemas, 2003). These problems have hindered the accurate evaluation of species-specific estimates of salmonid predation, and new approaches for assessing the occurrence of salmon and sea trout in phocid diets are needed to augment existing methodologies. In this study, a molecular genetic technique was used to identify salmonid prey species using the digested prey DNA present in the phocid scats.

DNA-based species diagnostic tests provide valuable information for both conservation and management of a variety of species, particularly where morphological traits cannot be used for species identification. The use of molecular genetic techniques for differentiating between closely related and congeneric species has been demonstrated for a variety of fish species, including salmonids (McGowan & Davidson, 1992; Pendas *et al*., 1995; Rehbein, Mackie & Pryde, 1995; Russell *et al*., 2000; Hold *et al*., 2001; Greig, Robertson & Banks, 2002; Purcell *et al*., 2004). Direct DNA sequencing of targeted regions has been widely used to provide unambiguous species identity for numerous taxonomic groups. For projects intending high throughput, however, sequencing can become very expensive. The popularity of alternative techniques that exploit DNA polymorphisms, such as random amplified polymorphic DNA (RAPD), single-stranded conformational polymorphism (SSCP), and restriction fragment length polymorphism (RFLP), has intensified owing to a combination of reliability, and relatively rapid and inexpensive sample screening. Many of these approaches have been based on highly conserved regions of the mitochondrial (mtDNA) genome such as the cytochrome *b* gene, and the 5S and 16S ribosomal RNA genes. These conserved regions facilitate the development of oligonucleotide primers that can be used across a wide taxonomic range (Meyer, 1994), yet possess sufficient diversity to detect species-level genetic differences. Moreover, the multiple copies of mtDNA present in each cell render it ideal for use in circumstances where the amount of sample DNA isolated is often of low quality and quantity, a common characteristic of faecal DNA.

Faecal genetic analysis is a powerful non-lethal technique that can be used to obtain information both on the identity and population genetics of the predator (Reed *et al*., 1997; Taberlet *et al*., 1997; Wasser *et al*., 1997; Hansen & Jacobsen, 1999; Parsons *et al*., 1999; Parsons, 2001), as well as information on prey and diet composition (Hoss *et al*., 1992; Poinar *et al*., 1998; Hofreiter *et al*., 2000; Jarman, Gales *et al*., 2002; Orr *et al*., 2004; Purcell *et al*., 2004). To date, however, genetic identification of pinniped prey species has concentrated on extracting DNA from physical prey remains (Orr *et al*., 2004; Purcell *et al*., 2004). Although this method is invaluable for identifying prey remains that lack species-specific structures (Orr *et al*., 2004), it is not applicable to faecal samples that are lacking undigested prey remains, and cannot detect prey items not represented by skeletal structures. Genetic identification of food species from the faecal matrix of large vertebrates has proved valuable for large cetacean species (Jarman, Gales *et al*., 2002; Jarman, Deagle & Gales, 2004), penguins (Jarman, Gales *et al*., 2002), and bears (Hoss *et al*., 1992), but to the best of our knowledge has not yet been applied to the study of pinniped diet. In this study, a polymerase chain reaction (PCR)-RFLP technique was developed to enable rapid differentiation among Atlantic salmonid prey species from DNA extracted directly from the matrix of phocid seal scats.

METHODS

Development and validation of salmonid markers

Direct sequencing of a 123 base pair (bp) segment of the mitochondrial cytochrome *b* gene has identified a minimum difference of 5.2% between the nucleotide sequences of sea trout *Salmo trutta* and Atlantic salmon *Salmo salar* (McVeigh, Bartlett & Davidson, 1991; McGowan & Davidson, 1992). PCR primers for amplification of this variable cytochrome *b* region (Fig. 1), and the 16S rRNA gene were developed using published sequences for both grey seal *Halichoerus grypus* (Genbank NC-001602) and Atlantic salmon (Genbank AF133701) mitochondria, and the cytochrome *b* sequence for sea trout (Genbank M64918).

Mitochondrial genome sequences were used to develop group-specific PCR primers that amplified salmonid DNA, and not phocid DNA. Sequences were aligned using the software CLUSTALW (Higgins, Fuchs & Bleasby, 1992) to identify regions around the desired salmonid variable cytochrome *b* gene that were both suitable for PCR primers and sufficiently variable to prohibit annealing and co-amplification of a homologous fragment in the pinniped genome. The primer pair salmcytb7 (5′-ATC TCC CAG CAC CAT CTA ACA T-3′) and salmcytb8 (5′-CGA AGG CAG TTA TTA TAG TGA GAA G-3′) was designed to amplify the desired 327 bp fragment (Fig. 1) using the primer design software OLIGO. The primer pair salm16s7 (5′-CAA GTA AAA ACG CAG TGA CC-3′) and salm16s18 (5′-TTG ATC GGC GTG ATG C-3′) were designed to amplify a 162 bp species-specific region in the 16S rRNA gene of *S. salar*. Primer sites was chosen to encompass the salmonid species-specific region, while avoiding co-amplification of phocid DNA and keeping the amplified fragment short enough to be useful for amplification of degraded faecal DNA.

The cytochrome *b* oligonucleotide primers were used in a PCR-RFLP assay. PCR conditions were optimized using pure salmon and sea trout DNA, and DNA extracted from two harbour seal *Phoca vitulina* blood samples. The cytochrome *b* amplification reactions were performed using 0.75 mM $MgCl_2$, 1 × NH_4 buffer, 0.2 mM of each nucleotide, 0.25 μM of each primer, 0.25 unit of *Taq*

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S. trutta   ---------ATCTCCCAGCACCATCTAACATCTCAGTTTGATGAAACTTT
S. salar    CACTAGTCGATCTCCCAGCACCATCTAACATCTCAGTTTGATGAAACTTT
H. grypus   CATTTATCGACCTACCCACACCGTCAAATATCTCAGCATGATGAAACTTT
                      ** **  **** ** ** *******  ************

S. trutta   GGCTCACTCTTAGGCTTATGTCTAGCCACCCAAATTCTTACCGGACTCTT
S. salar    GGCTCACTCTTAGGCCTATGTCTAGCCACCCAAATCCTTACCGGGCTCTT
H. grypus   GGATCTCTTCTCGGAATCTGCCTAATCCTACAGATCTTAACAGGCTTATT
            ** ** **  * **  * ** ***  *   ** **  * ** **  * **

S. trutta   CCTAGCCATACACTACACCTCCGATATCTCAACAGCCTTTTCCTCTGTTT
S. salar    CCTAGCCATACACTACACCTCCGATATCTCAACAGCTTTTTCCTCTGTTT
H. grypus   CCTAGCCATACACTACACCTCAGACACAACCACAGCCTTCTCATCAGTAA
            ********************* ** *   * ***** ** ** ** **

S. trutta   GCCACATTTGCCGAGATGTTAGCTACGGCTGACTCATCCGAAACATTCAC
S. salar    GCCACATTTGCCGAGATGTTAGCTATGGCTGACTCATCCGTAACATTCAC
H. grypus   CCCACATCTGCCGAGACGTAAACTACGGCTGAATCATCCGTTATCTTCAC
             ****** ******** ** * *** ****** *******  *  *****

S. trutta   GCTAACGGAGCATCTTTCTTCTTTATCTGTATTTATATACATATCGCCCG
S. salar    GCTAACGGAGCATCTTTCTTCTTTATCTGTATTTATATACACATCGCCCG
H. grypus   GCAAATGGAGCTTCCATATTCTTCATCTGCCTATACATGCATGTGGGACG
            ** ** ***** **  * ***** *****  * ** ** **  * *  **

S. trutta   AGGACTCTACTATGGTTCCTACCTATATAAAGAAACCTGAAATATCGGAG
S. salar    AGGACTTTATTATGGTTCCTATCTATATAAAGAAACCTGAAATATCGGAG
H. grypus   AGGACTATATTACGGCTCTTACACATTCACAGAGACATGAAACATCGGCA
            ****** ** ** ** ** **   **  * *** ** ***** *****

S. trutta   TTGTACTTCTACTTCTCACTATAATAACTGCCTTCG
S. salar    TTGTACTTCTACTTCTCACTATAATAACTGCCTTCG
H. grypus   TTATCCTCTTATTCACCATCATAGCCACGGCATTCA
            ** * **  ** *  **  ***  ** ** ***
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Fig. 1. Aligned grey seal *Halichoerus grypus* and salmonid (*Salmo salar* and *S. trutta*) cytochrome *b* sequences. Grey shading, cytochrome *b* primers; underlined, the 123 bp salmonid species diagnostic region; open boxes, *Alu*I restriction sites (AG|CT); dashes, unknown sequence; asterisks (*), nucleotide identity among all three sequences.

polymerase, and 10 ng of template DNA in a total volume of 10 μl. Cycling conditions included 1 min of denaturation at 92 °C, followed by 34 amplification cycles at 58 °C (45s at 92 °C, 45s at 58 °C, 1 min at 72 °C) and a final 2 min extension step at 72 °C. Species-specific banding patterns (restriction sites indicated in Fig. 1) were obtained by incubating 3–4 μl of PCR products with 1 unit of the restriction enzyme *Alu*I, in a 10 μl reaction at 37 °C overnight. The restriction products were separated on a 1.5% agarose gel containing ethidium-bromide and visualized by ultraviolet irradiation (Fig. 2). Sequence variation was confirmed between variable profile PCR products by direct sequencing of Qiaquick (Qiagen) purified PCR products.

In addition to the cytochrome *b* marker, the 16S rDNA marker specific to *Salmo salar* provided additional confirmation of the presence of Atlantic salmon in the template DNA of screened samples. PCR conditions were optimized using the same monospecific DNA samples as above, and all 16S rDNA amplifications were performed using 1.0 mM $MgCl_2$, 1 × NH_4 buffer, 0.2 mM of each nucleotide, 0.25 μM of each primer, 0.25 unit of *Taq* polymerase, and 10 ng of template DNA in a total volume

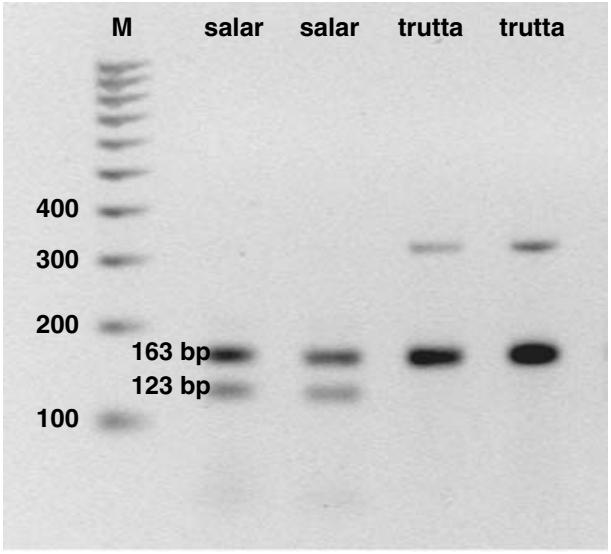

Fig. 2. A 1.5% agarose gel showing the species-specific cytochrome *b* RFLP banding pattern for Atlantic salmon *Salmo salar* and sea trout *S. trutta*. M, a molecular size ladder with band sizes (bp) as indicated.

of 10 μl. The 162 bp fragment was amplified using a 10 °C 'touchdown' procedure (Don *et al*., 1991), whereby after an initial 3-min denaturation step at 90 °C, 20 cycles of PCR were performed, each cycle consisting of 30 s denaturation at 91 °C, and 30 seconds of annealing starting at 65 °C and dropping by 0.5° per cycle. A final 15 cycles consisted of 30 s denaturation at 90 °C and 30 s annealing at 55 °C.

Field validation of molecular assay

To examine the effectiveness of the salmonid PCR-RFLP assay when confronted with the multiple sources of DNA present in faecal samples (i.e. fish, seal, bacteria, etc.), the molecular assay was validated using faeces collected from a captive-fed grey seal. The single adult female grey seal was housed in an outdoor concrete pool at the Gatty Marine Laboratory (St Andrew's, U.K.). Scat samples were collected from a seal that had been maintained on a monospecific diet of Atlantic salmon for 14 days. When the pool was drained and cleaned, scats were sampled using sterile wooden spatulas, preserved in an equal volume of 20% DMSO/NaCl, and stored at -20 or -80 °C. Samples from 7 different scats (≤ 1 week since the time of defecation) were collected. An additional 5 scat samples were collected from a juvenile female grey seal, housed in a separate pool and maintained on a diet consisting exclusively of herring to provide negative controls.

Two sub-samples were removed from each of the 12 scats after being preserved for an average of 381 days post-defecation. The scat sub-samples were removed randomly from the frozen, preserved faeces, and no hard prey remains were present in any of the samples removed for extraction. Total DNA was extracted from each sub-sample ($n = 24$) using the DNAce spin stool extraction kit (Bioline cat. BIO-28050). Approximately 0.30 g of frozen faeces was used in each faecal extraction, and genomic DNA was eluted in a final volume of 100 μl using the elution buffer (10 mM Tris-HCl, 1 mM EDTA). To avoid cross-contamination of faecal samples, sterile protocols were observed and an ethanol-wiped, flamed stainless steel spatula was used to transfer all faecal material. Furthermore, disposable laboratory glassware and pipette filter tips were used throughout the extraction procedure, and negative controls (that contained no faeces) were simultaneously processed to provide confirmation of a contamination-free environment.

Molecular screening of faecal DNA

The use of the salmonid mtDNA markers for screening seal scats for the presence/absence of salmon and sea trout was validated using the faecal DNA extracted from the captive grey seal scats. All samples were screened 'blind' to evaluate the PCR-RFLP approach using faecal DNA. The 24 faecal DNA extracts were re-numbered (the 'key' linking the screening numbers with the original extract numbers was held by an independent person) and screened using both the cytochrome *b* PCR-RFLP assay and the 16S rRNA PCR. The cytochrome *b* assay was performed twice on each faecal extraction to verify reproducible results. Both positive (salmon and sea trout DNA) and negative (seal DNA and no genomic DNA) control reactions were included in each PCR set. Owing to the low quantity of prey DNA present in each scat extract, it was necessary to increase the template volume in the PCR-RFLP assay. To obtain sufficient PCR product from faecal extracts to allow either *Alu*I restriction, or direct sequencing, 4 μl of extracted genomic DNA was used in each PCR. Likewise, 7 μl of the faecal PCR product (compared to 3 μl of salmonid template PCR product) was subsequently used in the 10 μl *Alu*I restriction digest. Quantification of the salmonid template DNA used in each PCR reaction was not performed because the multiple sources of DNA present in faecal DNA extracts prohibits accurate estimation of the quantity of DNA for a single prey species.

RESULTS

The cytochrome *b* fragment amplified from four of the seal faecal DNA extracts, two Atlantic salmon samples (positive control) and two sea trout samples (positive control) were purified and sequenced in both directions to confirm identity of the amplified product. Alignment of the resulting sequences confirmed that the cytochrome *b* amplicon from the faecal DNA was indeed salmonid, and not phocid, in origin.

The 16S rRNA primers amplified a fragment of the expected size (~ 162 bp) in the PCR reactions containing *S. salar* template DNA. The absence of amplified products from PCR reactions containing either *S. trutta* or *H. grypus* template DNA confirmed the species-specific nature of this small mitochondrial DNA marker.

Screening of all 24 faecal DNA extracts suggested variability in the amount of amplifiable salmonid DNA present in the seal faecal samples, as indicated by the band intensity of electrophoresed PCR products. Although the quantity of amplified product affected the ability to visualize RFLP bands, the presence of salmon in the diet of the seal as indicated by both the cytochrome *b* and 16S rRNA PCR products was accurate in 97.1% ($n = 69$ unambiguous PCR-RFLPs) of reactions. Moreover, if presence or absence of salmonid DNA were determined by a minimum detection rate of four out of six diagnostic assays, all 12 scat samples would be correctly identified for presence or absence of salmonid prey. Only one of 24 (4.2%) faecal extracts erroneously tested negative for the presence of salmon in two of three assays despite being fed a salmon diet (Table 1). No false positive results were obtained (Table 1).

DISCUSSION

Development of group-specific mitochondrial PCR primers, and a PCR-RFLP assay that produces salmonid

Table 1. Seal faecal DNA sample information and the presence (+) or absence (−) of salmon in the seal diet as indicated by the PCR results. CYTb 1 and 2, two independent cytochrome *b* PCR-RFLP assays; 16S, presence or absence of a 16S rRNA amplicon; ?, an ambiguous PCR result; grey shading, DNA extract for which false negative results were obtained in ≥ 2 PCR reactions

		Sample nos	Salmonid PCR results		
Seal diet	Scat no.	Extraction no.	CYTb 1	CYTb 2	16S
Salmon	1	5	+	+	+
		6	+	+	+
Salmon	2	7	+	+	+
		8	+	+	+
Salmon	3	9	+	+	+
		10	+	+	+
Salmon	4	11	+	+	?
		12	+	+	+
Salmon	5	13	?	+	+
		14	+	+	+
Herring	6	15	−	−	−
		16	−	−	−
Salmon	7	17	+	+	+
		18	+	−	−
Salmon	8	19	+	+	+
		20	+	+	+
Herring	9	21	−	−	−
		22	−	−	−
Herring	10	23	?	−	−
		24	−	−	−
Herring	11	25	−	−	−
		26	−	−	−
Herring	12	27	−	−	−
		28	−	−	−

species-specific banding patterns have proved both useful, and accurate, for determining the presence of Atlantic salmon in the diet of phocids. Although comparison of faecal DNA from seals maintained on salmon and sea trout diets was not possible owing to captive sampling constraints, the easily distinguishable RFLP banding pattern for these two congeneric salmonids suggests that identifying the presence of these two species from faecal DNA is feasible. In addition, the 16S rDNA fragment specific to Atlantic salmon provides rapid corroboration of the cytochrome *b* results when the presence of *S. salar* is indicated.

Both of the salmonid fragments amplified by PCR in this study were relatively small (< 400 bp) mitochondrial DNA sequences. These regions were targeted to maximize the detectability of prey DNA amongst the multiple sources of degraded DNA present in seal faeces. Previous non-invasive genetic studies (Kohn & Wayne, 1997; Farrell, Roman & Sunquist, 2000), and studies of the detectability of prey DNA among the predator gut contents (Zaidi *et al*., 1999) suggest that, owing to the DNA degradation caused by digestion enzymes, smaller sequences and multiple-copy DNA are easier to detect than larger, single copy DNA sequences. Despite the 165 bp difference between the cytochrome *b* and the 16S rRNA fragments, no difference in our ability to amplify these two fragments was detected.

Overall, the number of false negative results was very low, and only one DNA extract from a single scat sample did not contain enough salmon DNA to enable detection by PCR. Although the observed rate of PCR failure is considerably lower than other studies incorporating analysis of faecal DNA (Reed *et al*., 1997; Parsons, 2001), false negative results are relatively common when attempting to amplify DNA from faeces, and may be the result of either the age of the scat or the quantity of scat collected. Due to the behaviour of the captive seal, it was not possible to collect scats on a daily basis and the scats used in this study were all collected on the same day. Consequently, some of the scats may have been as much as 1 week old (time elapsed between pool-draining/cleaning sessions). Prey detection heterogeneity among sub-samples within a scat was also apparent in at least one of the scats sampled. Further analysis involving the collection of scats on a daily basis would allow direct testing of the effect of time-since-defecation on prey detectability. In addition, collection of the entire scat followed by homogenization and sub-sampling may eliminate errors due to the proportion of the scat collected.

The captive diet constraints prevented testing of this technique on mixed-diet faecal samples, and thus conducting an extensive validation of the technique on emulated wild seal faecal samples. In lieu of this, the nucleotide-nucleotide BLAST search engine, available on the web (http://www.ncbi.nlm.nih.gov/BLAST/), was used to assess non-specific matches to the salmonid primers. The 22-mer forward cytochrome *b* primer had complete complementarity with nine species of bony fishes including several *Salmo* sp., as well as *Rhodeus ocellatus* (rosy bitterling), and the salmonids *Salmothymus ohridana* and *Acantholingua ohridana*. There was some complementarity with bird (e.g. *Tangara fastuosa*, *Passerina amoena*) and mammal (*Canis aureus*, *Saguinus fuscicollis*) species, but the likelihood that these items would be present in the gut of *Phoca vitulina* or *Halichoerus grypus* to cause false positive results is negligible. No species other than *Salmo salar* had complete identity with the 25-mer reverse cytochrome *b* primer. Of the 13 species that shared sequence identity with the 16S forward primer, all were salmonids except for Arctic char *Salvelinus alpinus*. None of these species, however, shared identical sequences with the 16S reverse primer. DNA extracted from mammalian faeces may comprise numerous potential sources of DNA, including both primary and secondary prey, mammal and bacterial DNA. The stringent design of the PCR primers and conditions, however, should prohibit amplification from orthologous genes and primer mismatch.

The use of molecular techniques to characterize diet composition has proved useful for a variety of predator species (Symondson, 2002). PCR-based techniques have proved effective not only in identifying prey remains from vertebrate faeces (Purcell *et al*., 2004) and bird pellets (Taberlet & Fumagalli, 1996), but also from DNA

extracted from faeces or intestinal contents in the absence of recognizable prey remains (Rosel & Kocher, 2002; Symondson, 2002). The PCR-RFLP approach adopted in this study is a relatively inexpensive and rapid method suitable for other projects that require high throughput screening for species detection. Both the methods and PCR primers presented here may prove useful to studies of foraging habits and dietary preferences of a wide range of both phocid and otariid pinnipeds, as well as other mammalian piscivores.

The use of DNA amplified from the scat matrix opens up important new possibilities for studying the occurrence of those prey species that may be underrepresented in pinniped diet as reconstructed from hard prey remains. Identifying the presence of *S. salar* and *S. trutta* in the diet of pinnipeds around Britain could prove a valuable addition to studies examining the interactions between seals and salmon fisheries. Moreover, molecular identification of the presence of these two species in scats collected from wild seals could provide additional information for parameterizing models of the impact of seal predation on salmon stocks. At present, however, the use of DNA is not a replacement for conventional hard parts analysis, but rather an additional tool to augment scat analyses. Future work integrating both direct (species identification of prey remains) and indirect (molecular prey identification) techniques for concurrent screening of the same scat samples has the potential to significantly enhance our understanding of marine predator–prey interactions.

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