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Utilizing next-generation sequencing to identify prey DNA in western North Atlantic grey seal *Halichoerus grypus* diet

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ABSTRACT: Increasing grey seal *Halichoerus grypus* abundance in coastal New England is leading to social, political, economic, and ecological controversies. Central to these issues is the foraging ecology and diet composition of the seals. We studied grey seal feeding habits through next-generation sequencing of prey DNA using 16S amplicons from seal scat (n = 74) collected from a breeding colony on Monomoy Island in Massachusetts, USA, and report frequency of occurrence and relative read abundance. We also assigned seal sex to scat samples using a revised PCR assay. In contrast to current understanding of grey seal diet from hard parts and fatty acid analysis, we found no significant difference between male and female diet measured by alpha and beta diversity. Overall, we detected 24 prey groups, 18 of which resolved to species. Sand lance *Ammodytes* spp. were the most frequently consumed prey group, with a frequency of occurrence (FO) of 97.3%, consistent with previous studies, but Atlantic menhaden *Brevoortia tyrannus*, the second most frequently consumed species (FO = 60.8%), has not previously been documented in US grey seal diet. Our results suggest that a metabarcoding approach to seal food habits can yield important new ecological insights, but that traditional hard parts analysis does not underestimate consumption of Atlantic cod *Gadus morhua* (FO = 6.7%, Gadidae spp.) and salmon *Salmo salar* (FO = 0%), 2 particularly valuable species of concern.

KEY WORDS: Foraging ecology · Prey DNA · Molecular scatology · Next-generation sequencing · Diet · *Halichoerus grypus* · Grey seal

1. INTRODUCTION

The presence of seals in coastal communities often incites conflict with fishing activity, as the impacts of pinnipeds on fisheries stocks remains uncertain in many cases. The commercial fishing industry, particularly groundfishing, has played an important role in the economy of New England for over 400 yr (Murawski mid-1990s). During the past decade (2006–2016), 3.1 billion metric tons of fish has brought New England $11.8 billion in revenue (NOAA Fisheries 2019). As the value of the fishing industry has been increasing over time, so has the population of grey seals *Halichoerus grypus* in New England (Waring et al. 2016). Over a similar time frame (1994–2011), grey seal counts increased from 2010 individuals (Rough 1995) between Muskeget and Monomoy Islands (MA, USA) to an estimated 17 060 individuals based on digital surveys (Moxley et al. 2017). The increased abundance of grey seals increases the likelihood of interactions between seals and fisheries. Among the most concerning of potential interactions according to fishermen in Cape Cod are seals predating commercially important fish stocks, depreda-

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tion on important bait fish stocks, and destruction of fishing equipment (Gruber 2014).

In addition to the social tension between fisheries and grey seals, overfishing and rebounding seals have undoubtedly altered the Gulf of Maine ecosystem through specific predator–prey interactions. The diet of grey seals is understudied in the USA (but see Ampela & Ferland 2006, Ampela 2009, Lerner et al. 2018), as most research has been conducted in Canada and the UK (Bowen et al. 1993, Bowen & Harrison 1994, Hammond et al. 1994, McConnell et al. 1999). Grey seal diet is known to vary with prey availability and assemblages (Bowen et al. 1993, McConnell et al. 1999), which may differ between US and Canadian waters.

Grey seal diet is known to differ between males and females in both the USA and Canada. Male grey seals weigh approximately 1.6 times more than females, and therefore require more food for maintenance (Beck et al. 2005, 2007). Males store energy in preparation for fasting during the breeding season, while females utilize extra resources during both pregnancy and the production of high-fat milk (Beck et al. 2005, 2007). The sexes also exploit separate habitats for foraging; males tend to forage offshore, while females forage inshore (Breed et al. 2006). Given the physical and behavioral differences between males and females, differences in diet would be expected. As hypothesized, Beck et al. (2005, 2007) found, through fatty acid analysis, that Canadian grey seal males consumed a greater diversity of species and more energetically dense species than females. Ampela (2009) detected significant overall diet differences between the sexes, through both hard parts and fatty acid analysis. Using multiple methods of dietary investigation to clarify the effects of variables, including sex, is critical to understanding the niches and environmental impacts of consumers. Further, accurate information regarding grey seal diet is necessary to resolve social, political, and environmental conflicts (Reed et al. 1997, Ampela 2009, Phillips & Harvey 2009).

A common technique utilized to study pinniped diets is hard parts analysis from scat and stomach contents, which involves identifying structures (otoliths, lenses, beaks, exoskeletons, etc.) that survive digestion and provide information about prey items consumed within hours or days (Prime & Hammond 1990, Bowen & Harrison 1994). Other methods include fatty acid analysis (Beck et al. 2005, 2007, Ampela 2009) and stable isotope analysis (Lerner et al. 2018) of blubber, whiskers, or fur, both of which have the potential to detect prey items that have been consumed over weeks to months. Of the methods, hard parts analysis from scat samples allows for the simplest, least expensive, and minimally invasive collection of sampling units. However, the hard parts methodology is limited by a number of well-known biases (Jobling & Breiby 1986, Prime & Hammond 1990, Bowen et al. 1993), including the prerequisite that identifiable hard parts are present. Hard parts may not be present if (1) the hard parts are completely digested (Bowen 2000); (2) the seal tears off pieces of soft tissue from the prey item without consuming any hard parts (Phillips & Harvey 2009); or (3) the seal consumes prey items which lack hard parts that can be identified to species (e.g. skates; Pierce & Boyle 1991).

Molecular scatology, the identification of DNA in scat, has been applied to diet studies of a variety of taxa, including birds (Oehm et al. 2011), bears (Höss et al. 1992), bats (Clare et al. 2009), wild cats (Farrell et al. 2000), and pinnipeds (Deagle & Tollit 2007, Deagle et al. 2009, Emami-Khoyi et al. 2016; for reviews see Symondson 2002, Waits & Paetkau 2005). A scat-based approach is particularly beneficial for pinniped diet studies, as a semi-aquatic lifestyle means that these large animals can be elusive and difficult to capture and restrain for invasive sampling. Sampling scat also allows for increased sample sizes, since catching and restraining many individual pinnipeds is not practical. The use of a DNA-based analysis of pinniped scat is also valuable because it offers an opportunity to address the biases associated with hard parts analysis. Though the hard parts of prey items may be impossible to recover and identify in some cases, consumption of any prey item (partial or complete) entails consumption of cell-bound DNA. Molecular-based techniques face disadvantages as well (such as recovering degraded DNA and reducing effects of molecular inhibitors; see Taberlet et al. 1999), but molecular scatology permits an analysis of prey contents via scat without the biases of larger physical remains. In addition, molecular scatology can be used to identify the sex of the animal depositing scat by isolating DNA fragments located on sex chromosomes (Morin et al. 2005, Matejusová et al. 2013).

Our objectives were to (1) assess grey seal diet using a novel approach for this system (molecular scatology via next-generation sequencing) and (2) determine if sex of the seal is an important factor influencing US grey seal foraging ecology from a genetic perspective. To determine the composition of grey seal diet at primarily the species level, we identified prey DNA present in scats collected from haul-

out sites along Monomoy Island, MA, USA. To quantitatively study importance of seal sex to the diet, we investigated statistical differences in alpha diversity (i.e. prey richness) and beta diversity (i.e. prey group composition between samples; Anderson et al. 2011) between males and females using frequency of occurrence (FO) data. We also examined relative read abundance (RRA) data for all prey groups between seal sexes as a proxy for prey biomass in the diet (see Deagle et al. 2019 for discussion).

2. MATERIALS AND METHODS

2.1. Sample collection and DNA extraction

Wild grey seal *Halichoerus grypus* scats (n = 84) were collected on 17 May 2017 from the beaches of Monomoy Island (41.55° N, 70.00° W) off of Cape Cod, MA (Fig. 1). Most scats (~70) were considered fresh, as assessed by high moisture content and the presence of hauled out grey seals where samples were collected. The remaining scat samples (~14) were mostly, if not entirely, dried out and not considered fresh. It is unlikely that any duplicate scats were collected from any one individual, as there were hundreds of seals present and we moved systematically in one direction down the beach to ensure no areas were collected from twice. Additionally, most scats were freshly deposited, and an individual seal is unlikely to have defecated multiple times in our short collection period.

Scats were placed into either Ziploc® or Whirl-Pak® bags and held on ice until arrival at the University of New England (Biddeford, ME). DNA subsamples, approximately 5 ml, were taken randomly from manually homogenized (mixed via wooden sticks) scats, placed in 100% ethanol, and stored at −20°C until extraction. Samples were prepared for extraction by decanting the excess ethanol, absorbing residual ethanol in the sample with a Kimwipe®, and evaporating any remaining ethanol at room temperature until the samples were visually dry. Approximately 200 mg of scat were isolated from each sam-

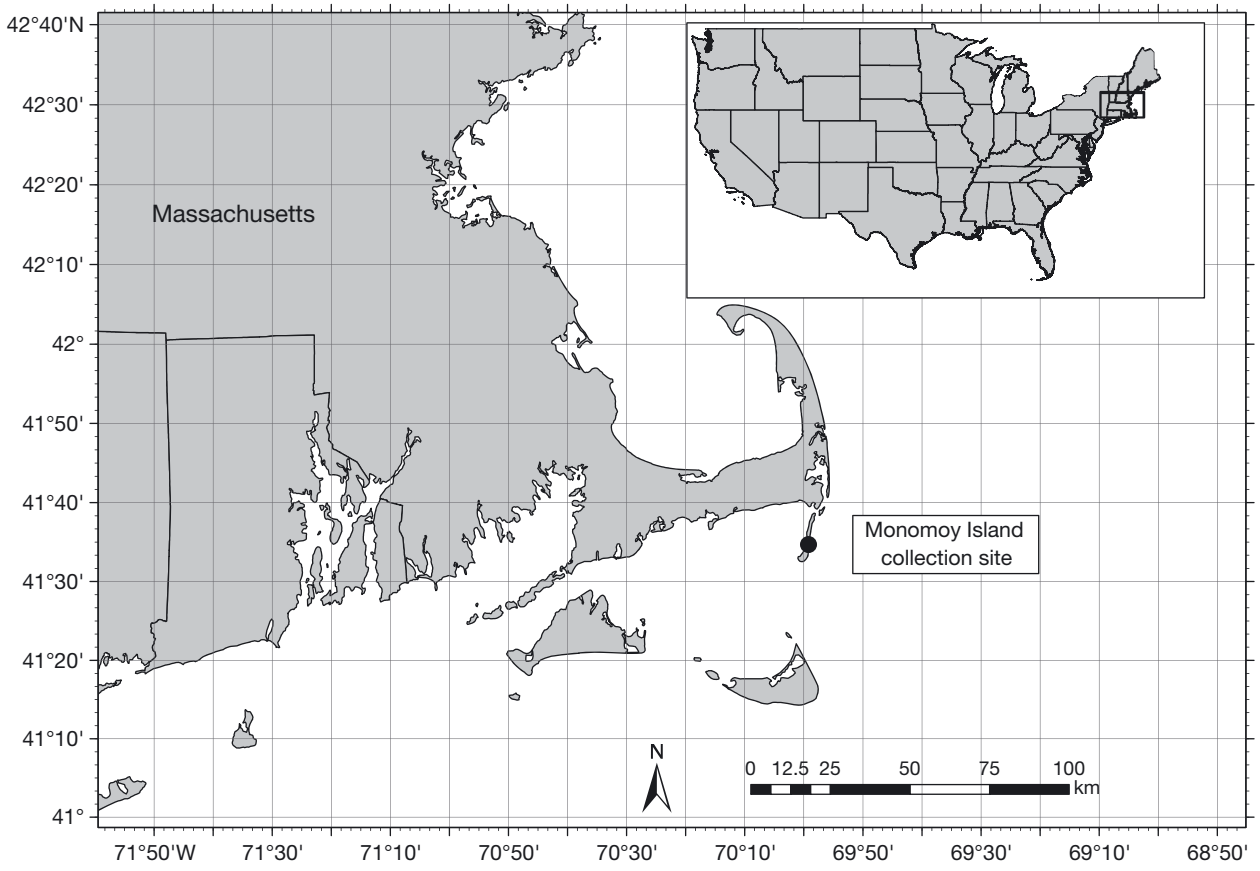

Fig. 1. Cape Cod, Massachusetts (inset map shows this region in context of the USA) highlighting Monomoy Island and the approximate location where grey seal scats were sampled (black circle; 41.6° N, 70.0° W). Map created in ArcGIS Pro 2.5.0 using modified 'US State Boundaries' layer data (Sources: US Census, ESRI Training Services)

ple for extraction. Gloves were changed regularly, and each sample was handled with separate, single-use disposable items (e.g. cups for evaporating, Kimwipes® for excess ethanol), and flame-sterilized equipment.

Extractions were conducted using QIAamp® DNA Stool Mini Kit (QIAGEN), where the initial incubation period took place overnight (~24 h at 60°C), and 1 µl of carrier RNA was added to each sample alongside 600 µl ethanol after the second incubation. Genomic DNA was eluted into a final volume of 100 µl. Extraction blanks, where all reagents were used with no sample input, were included to monitor for cross-contamination. Additionally, scat extractions took place in a separate building than any prey tissue extractions and all amplification steps to reduce contamination (Taberlet et al. 1999).

2.2. PCR identification of seal sex

We utilized primers from Matejusová et al. (2013) to amplify a fragment of the ZFY gene (69 bp; F_5'-GCA AGC TCC GAG ATT AAA CCA-3', R_5'-TGA TCT AGC AGC TAA ATT GCT ATC G-3') and primers (Hg6.1) from Allen et al. (1995) to amplify a microsatellite locus as an internal control for DNA quality and amplification success (150–166 bp; F_5'-TGC ACC AGA GCC TAA GCA GAC TG-3', R_5'-CCA CCA GCC AGT TCA CCC AG-3'). The ZFY and Hg6.1 primers were used in a multiplex PCR (cycling conditions: denature at 95°C for 3 min, 45 cycles of 95° for 15 s, 60°C for 1 min, 72°C for 30 s, final extension of 72°C for 10 min) to identify grey seal sex for each wild scat (male and female banding patterns shown in Fig. 2). To verify the success and optimize the performance of these primers, we tested them using scats from 3 grey seals of known sexes (1 male, 2 females) collected at the Smithsonian Institution's National Zoological Park. Each PCR (10 µl) contained 6.15 µl ddH_20, 2.0 µl 5× MyTaq™ reaction buffer, 0.25 µl primer Hg6.1 forward and reverse combined (10 µM), 0.5 µl ZFY forward and reverse combined (10 µM), 0.1 µl MyTaq™ DNA Polymerase, and 1 µl template (scat-derived) DNA extract.

To reduce the probability of identifying a true male as a female (Reed et al. 1997, Matejusová et al. 2013), we required a positive microsatellite band and a negative ZFY band across 3 reactions to designate a sample as female. If a ZFY band was present in any replicate, the sample was designated as male. In cases where the initial PCR reaction failed, the sample was tested twice more. Samples that failed all 3

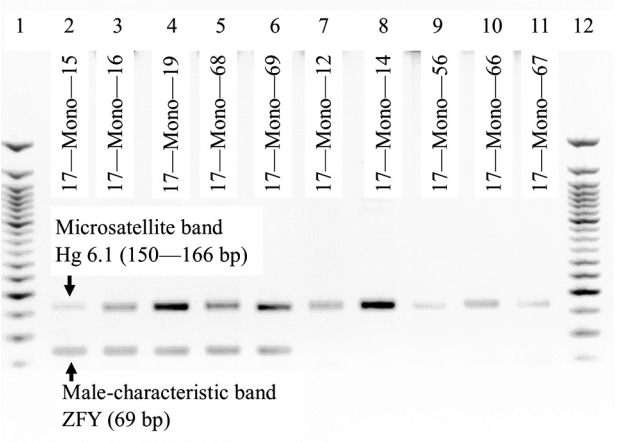

Fig. 2. Ethidium-bromide stained 2% agarose gel (color inverted) illustrating male and female banding patterns. The outermost lanes (1 and 12) contain a 50 bp ladder. Lanes 2–11 contain PCR product from wild grey seal scat in the 2017 Monomoy Island samples used in this study. The ZFY band (Matejusová et al. 2013) is the male-characteristic band, while the microsatellite band (Hg 6.1 from Allen et al. 1995) acted as a control band for DNA viability. Lanes 2–6 would be classified as male, and lanes 7–11 would be classified as female

reactions were diluted 1:5 (to dilute PCR inhibitors which may be impacting the reaction) and tested in duplicate again, then diluted to 1:10 and once more tested in duplicate. If no amplification was observed at this point, the sample was excluded from analyses (Fig. S1 in the Supplement at www.int-res.com/articles/suppl/m655p227_supp.pdf).

2.3. Sequencing for prey DNA analysis

We used primer set B (~110–130 bp) from Deagle et al. (2009) for our prey library (Chord_16S_FTagB_5'-[ATG] CGA GAA GAC CCT RTG GAG CT-3', Chord_16S_R_Short_5'-CCT NGG TCG CCC CAA C-3', where the bracketed nucleotides were tags utilized to differentiate primer A from B in Deagle et al. (2009) to amplify Chordata mtDNA 16S amplicons from each sample. Forward and reverse primers incorporated an additional Nextera-compatible adapter sequence for indexing (identification tags unique for each sample) prior to sequencing.

In the interest of assessing the taxonomic resolution achieved by primer set B with documented US grey seal prey, 1–5 sequences, as available, for each of 29 fish species known to occur in grey seal diets (Ampela 2009, Table S1 in the Supplement) were organized into haplotypes using FaBox v.1.41 (Villesen 2007, Fig. S2 in the Supplement). Because

Ampela (2009) noted the presence of skates (Rajidae), we included as many skate species in the Northeast Skate Complex as were available on GenBank (clearnose skate *Raja eglanteria*, thorny skate *Amblyraja radiate*, smooth skate *Malacoraja senta*). Ultimately, 96 sequences were condensed into 45 haplotypes (based on 100% sequence similarity), which were then aligned with the forward and reverse sequences of primer set B in BioEdit v.7.0 (Hall 1999) using ClustalW (Larkin et al. 2007). Of the 45 haplotypes, only 2 haplotypes included sequences from more than one species: family Gadidae included 4 Atlantic cod *Gadus morhua* sequences and one pollock *Pollachius virens* sequence, and family Pleuronectidae included 4 winter flounder *Pseudopleuronectes americanus* sequences and 1 yellowtail flounder *Pleuronectes ferruginea* sequence. All other haplotypes identified one species.

To validate primer set B (Deagle et al. 2009) for use in the western North Atlantic, the primers were tested against tissue-derived DNA (extracted using Zymo Research Genomic DNA™ Tissue MiniPrep kits according to manufacturer instructions) from a subset of prey species. The subset consisted of 7 chordate species chosen for high prevalence in grey seal diet (Ampela 2009) and for phylogenetic diversity: winter skate *Leucoraja ocellata*, winter flounder *P. americanus*, sand lance *Ammodytes americanus*, Atlantic cod *G. morhua*, fourspot flounder *Hippoglossina oblonga*, red hake *Urophycis chuss*, and windowpane flounder *Scophthalmus aquosus*. Longfin squid *Doryteuthis pealeii*, a non-chordate, was also included as a known grey seal prey item which was not expected to produce an amplicon. All 8 species produced the expected results upon visualization via an ethidium bromide-stained 2% agarose gel (Fig. 3), with anticipated fragment sizes observed for the chordate species and no amplicon visible for the non-chordate squid.

Target amplicons were produced from seal scat extractions via PCR reactions (25 µl) composed of: 14.8 µl ddH_20, 5.0 µl 5× MyTaq™ Reaction Buffer, 2.0 µl of 10 µM forward and reverse primer, 2.0 µl of 100 µM blocking primer (see Section 2.4), 0.2 µl MyTaq™ DNA Polymerase and 1 µl of template (scat-derived) DNA extract. Cycling conditions were 95°C for 15 min, 35 cycles of 95°C for 30 s, 57°C for 90 s, and 72°C for 30 s, followed by a final extension at 72°C for 10 min. Each wild grey seal scat DNA sample was tested up to 3 times for amplification, and a sample was considered a failure if no amplification was achieved. A 'sample' is hereafter considered the individual DNA extraction for each grey seal scat which

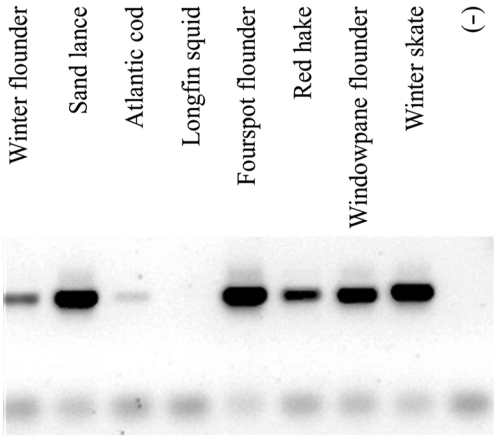

Fig. 3. Ethidium-bromide 2% agarose gel image (color inverted) representing the use of a universal chordate primer (primer set B; Deagle et al. 2009) on 8 potential grey seal prey species. Species were chosen based on prior evidence of grey seal predation and for phylogenetic diversity. Prey DNA was tissue-extracted and normalized to a concentration of 10 ng μl^{-1}. Primer B shows positive amplification for 7 of 7 chordates (longfin squid being the non-chordate and negative on a gel as expected)

amplified successfully or the extraction for each control condition. PCR amplicons were cleaned with a GeneJET™ PCR Purification Kit (Thermo Fisher Scientific) according to manufacturer protocols. Cleaned PCR products (15 µl) were then sent to the University of Minnesota Genomics Center (UMGC) for library preparation, sequencing, and post-sequencing cleaning. The UMGC utilized Nextera adapters and indexing primers for library preparation. Amplicons were sequenced using Illumina MiSeq ($1/8^{th}$ flow cell) with 2 × 300 bp chemistry.

Initial sequence cleaning steps were performed by the UMGC. PANDAseq software (Masella et al. 2012) was utilized to assemble forward and reverse reads with a primer match threshold of 0.6 (default). PANDAseq also removed primer and adapter sequences from reads. QIIME (Caporaso et al. 2010) was then used to subsample 5000 reads from each PCR sample to achieve a more even sequencing depth for comparisons across samples. Finally, de novo operational taxonomic units (OTUs) were identified based on 97% sequence similarity. The metabarcoding data processed to this point and used in the following analyses are available in the Dryad Digital Repository (https://doi.org/10.5061/dryad.bzkh1896s).

For the entire data set, we first identified the maximum number of copies for each OTU and trimmed those OTUs which had a maximum copy count of less than 10 (Thomsen et al. 2016), as they were pre-

sumed sequencing errors. For each sample, we then trimmed OTUs that comprised less than 1% of the total number of reads for that sample. Additionally, we excluded samples with fewer than 100 total prey reads from further analyses (McInnes et al. 2016). As indices of prey abundance, we calculated both FO and RRA. For FO, we considered a prey group 'present' if it comprised more than 1% of the total number of reads for a given sample and 'absent' if not. FO is reported as the number of samples in which a prey group was present. For RRA, we divided the number of reads of a prey group in a sample by the total number of reads in that sample. Overall RRA is reported as the average RRA of a prey group across all samples.

Final OTUs were taxonomically identified via a BLAST search (Altschul et al. 1990) against the GenBank database (Benson et al. 2008) of NCBI. The best hit for each OTU was determined based on the lowest 'Expect' value (e-value), with a minimum threshold of $1e^{-20}$ (following Thomas et al. 2014). In the event that multiple species assignments shared the same lowest e-value, those species that were geographically implausible were discarded and the species known to inhabit the Cape Cod region was chosen. In the event that 2 or more species known to inhabit the Cape Cod area shared the same lowest e-value, the OTU was assigned to family or genus rather than species. In a few cases, one flounder species was identified with a marginally lower e-value than other related flounders, but we approached flatfish conservatively due to underrepresentation in GenBank and classified such flounders to family. For one OTU, the only species identified by BLAST was a sea robin *Prionotus scitulus*, which is not found in New England waters. The common sea robin *P. carolinus*, which is found in New England and a documented food item of grey seals (Ampela 2009), is not represented in GenBank but was assumed to be the origin of the OTU. In another case, the best and geographically plausible hits for an OTU matched 2 species (*Stichaeus punctatus* and *Zoarces americanus*) in unrelated families. Because *S. punctatus* occurs infrequently in New England waters and has not been identified in US grey seal diet (Ampela 2009), we identified this OTU as more likely to be *Z. americanus*. Overall, identified OTUs were categorized as either (1) authentic prey reads, (2) prey items unique to captive controls, (3) predator reads (those originating from a seal), or (4) obvious contaminants. We constructed a species accumulation curve to assess whether we had a sufficient sample size from Monomoy to capture grey seal prey diversity; the presence of an apparent asymptote in the curve would indicate that our samples identified most consumed prey items.

2.4. Controls

Several control samples were also included in this study: (1) a PCR blank (negative control — no template), (2) a mix of tissue-extracted DNA from 4 fish species ('fish mix' positive control — 20 ng μl^{-1} of sand lance, skate *L. ocellata*, gadid *G. morhua*, and Pleuronectidae *P. americanus*), (3) 6 samples collected from female grey seals at the Aquarium of Niagara (Niagara Falls, NY, USA) with a known diet (to monitor accuracy), and (4) one duplicate of a wild sample (to monitor consistency).

Given that primer set B amplifies chordate DNA, both predator (seal) and prey (fish) DNA will amplify. Shedding of epithelial cells during digestion typically leads to a higher abundance of predator DNA compared to prey DNA in a scat produced by that predator (Symondson 2002). To suppress predator DNA amplification, a blocking primer (see Vestheim & Jarman 2008) was designed paralleling the protocol in Deagle et al. (2009) to block seal DNA: the first (5'end) 8 bases of the blocking primer overlap with the 3' end of the universal primer, the subsequent 25 bases are unique to the grey seal (GenBank Accession: X72004.1), and the end (3' end) position consists of a C3 spacer (see alignment of *H. grypus* blocker in Fig. S2). C3 spacers are 3 carbon chains which, when located at the 3' end of a DNA strand, prevent the incorporation of additional nucleotides by polymerase, thereby restricting amplification. To verify the efficacy of the blocking primer, a set of PCR reactions with the blocking primer (100 µM) and a set without the blocking primer were compared on a 2% agarose gel. Each PCR set contained one reaction with the 'fish mix' DNA (0.2 ng μl^{-1}) as a template and one reaction with tissue-extracted grey seal DNA (10 ng μl^{-1}).

2.5. Statistical analyses

All statistical analyses were performed in R v.4.0.1 (R Core Team 2020). Alpha diversity (i.e. prey richness) was defined as the total number of prey groups or species present in a sample (based on FO). Because our model violated parametric assumptions (Shapiro-Wilk normality test, $p = 0.00030$; Fligner-Killeen test of homogeneity of variances, $p = 0.015$), we used the Wilcoxon-Mann-Whitney test to investi-

gate the effect of seal sex on prey richness. Wilcoxon-Mann-Whitney tests were also performed on RRA data for each prey group between males and females to test for significant differences in number of reads.

Though there are a variety of measures used for beta diversity calculations (Koleff et al. 2003, Anderson et al. 2011), the Sørensen index (Sørensen 1948) was chosen because it accommodates presence–absence data and because it excludes joint species absences. The Sørensen index is a measure of similarity between samples, and dissimilarity was calculated as (1 − Sørensen). To analyze beta diversity, a site (sample) × species (or prey group) matrix was composed with presence (1) / absence (0) data (based on FO). A pairwise dissimilarity matrix, a matrix of Sørensen index dissimilarity (1 − Sørensen) for all possible pairs of samples, was then created with function 'betadiver' in the 'vegan' package (Oksanen et al. 2018). The resulting dissimilarity matrix was used to test whether dissimilarities significantly differed when samples from different sexes were compared. A permutational ANOVA (PERMANOVA) was implemented with the 'adonis' function (vegan) to model groups against the dissimilarity matrix with 999 permutations. Group dispersion was also modeled via 'betadisper' (vegan) to separate effects of dissimilarity measures from dispersion around the centroid (Warton et al. 2012). Although there are no options for post hoc analyses of 'adonis' models with presence–absence data, PERMANOVA modeling with a 'simper' (vegan) analysis can be used to investigate which prey species drive significant dissimilarities. 'Simper' calculates the contribution to dissimilarity of prey species which cumulatively explain 70% of dissimilarity between groups.

3. RESULTS

3.1. Wild sample viability and predator sex determination

For the Monomoy samples, 80 of 84 (95%) samples amplified successfully for the sex determination loci. The 4 unsuccessful samples were omitted from further analyses, as prey DNA would be unlikely to be recovered if predator DNA was not recovered. Our sex determination protocol correctly assigned seal sex (1 male, 2 females) from 3 scats collected from known grey seals *Halichoerus grypus* by the Smithsonian Institution's National Zoological Park. Of the 80 Monomoy samples, 25 were classified female and 55 were classified male.

3.2. Grey seal feeding habits

A total of 86 samples were included in the study: 77 wild grey seal scat samples (3 of the aforementioned 80 samples lacked template material for prey sequencing), one duplicate of a wild sample, 6 control samples from the Aquarium of Niagara, one positive 'fish mix' control, and one negative control. A total of 1 095 281 successful reads were produced from the Illumina MiSeq (1/8th flow cell) run for the samples in this study, with 371 346 reads after subsampling (mean sample^{-1}: 4317 reads; range: 3–4996 reads) organized into 4721 OTUs. After we trimmed the 4721 OTUs with a maximum copy count of less than 10, there were 364 308 reads organized into 78 OTUs. From the 78 OTUs, there were 31 taxa identified which were organized into 4 groups: (1) authentic prey items (n = 24), (2) prey items unique to captive controls (n = 3), (3) predator reads (n = 1), and (4) obvious contaminants (n = 3).

The 3 prey items unique to captive controls were *Clupea pallasii* (Pacific herring), *Mallotus villosus* (capelin), and *Osmerus mordax* (rainbow smelt). The predator reads were grey seal sequences. Three OTUs were classified as obvious contaminants: *Homo sapiens* (human; n = 10 samples), *Oscillibacter valericigene* (bacteria; n = 1), and *Lepomis macrochirus* (bluegill; n = 1). The bacteria sequence is outside of the intended Chordata scope of this primer set and was discarded. Although bluegill was identified, this species does not occur in New England and was not being studied in the lab. The bluegill OTU was discarded because they are unlikely to be a true prey item given that they are freshwater, inland fish with an easternmost range limit around New Hampshire (NatureServe 2013), approximately 250 km from Cape Cod.

The 24 authentic prey items and associated FO and RRA measures are shown in Table 1. Overall, the top 5 prey groups in order via FO analyses were *Ammodytes* spp. (sand lance), *Brevoortia tyrannus* (Atlantic menhaden), Pleuronectidae spp., *Scomber scombrus* (Atlantic mackerel), and *Prionotus carolinus* (northern sea robin). The top 5 prey groups via RRA were the same as those via FO, although in a slightly different order: sand lance, Atlantic menhaden, northern sea robin, Atlantic mackerel, and Pleuronectidae spp.

Overall, 3 of the 77 wild grey seal scat samples failed to produce any reads after all cleaning and filtering steps. Between the 74 samples remaining (n = 24 female, 50 male), 24 OTUs were identified

Table 1. Taxa identified in this study along with frequencies of occurrence (FO) and average relative read abundance (RRA) for western North Atlantic grey seal scat samples from Monomoy Island (n = 74). Also shown are FO values from Ampela (2009) (n/a: not available). The table is sorted from highest to lowest percentage of the diet by FO in this study

Scientific name (common name)	Index of abundance (this study) FO (%)	RRA (%)	Ampela (2009) FO (%)
Ammodytidae spp. (sand lance)	97.30	55.17	14.00
Brevoortia tyrannus (Atlantic menhaden)	60.81	14.40	n/a
Pleuronectidae spp.	25.68	3.43	3.00
Scomber scombrus (Atlantic mackerel)	22.97	3.79	1.10
Prionotus carolinus (northern sea robin)	21.62	5.10	n/a
Merluccius bilinearis (silver hake)	18.92	2.29	2.00
Urophycis chuss (red hake)	13.51	1.65	9.40
Urophycis regia (spotted codling)	13.51	0.92	n/a
Clupea harengus (Atlantic herring)	12.16	0.85	2.30
Zoarces americanus (ocean pout)	8.11	1.01	<1.0
Gadidae spp.	6.67	2.18	5.00
Alosa spp.	5.41	0.58	n/a
Peprilus triacanthus (Atlantic butterfish)	5.41	0.30	n/a
Myoxocephalus octodecemspinosus (longhorn sculpin)	4.05	1.08	2.50
Etropus microstomus (smallmouth flounder)	4.05	0.18	n/a
Scophthalmus aquosus (windowpane flounder)	4.05	0.23	7.10
Tautogolabrus adspersus (cunner)	2.70	0.66	n/a
Morone saxatilis (striped bass)	2.70	0.04	<1.0
Rajidae spp. (skates)	2.70	0.85	24.50
Alosa mediocris (hickory shad)	1.35	0.20	n/a
Alosa sapidissima (Atlantic shad)	1.35	0.38	n/a
Melanogrammus aeglefinus (haddock)	1.35	0.10	n/a
Paralichthyidae spp.	1.35	0.35	1.90
Hippoglossina oblonga (fourspot flounder)	1.35	0.02	1.90

(Table 1). Of those 24 OTUs, 18 achieved species-level resolution, one identified to genus, and 5 resolved to family level. From the species accumulation curve that was constructed for Monomoy samples (Fig. 4), the presence of an asymptote at approximately 75 samples indicates that sample size was sufficient to capture most grey seal prey species.

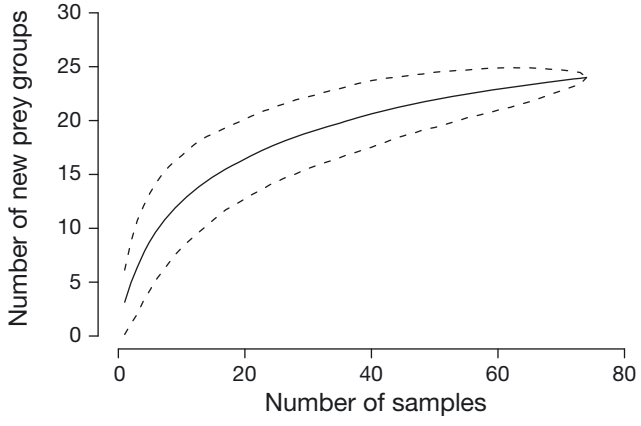

Fig. 4. Species accumulation curve (solid line) with 95% CIs (dashed lines) for grey seal scat samples collected from Monomoy Island during the spring of 2017. The presence of an asymptote near approximately 75 samples indicates that our sample size was sufficient to describe grey seal diet for this time and location

3.3. Controls

The negative control contained zero food item reads. The positive 'fish mix' control contained all 4 expected OTUs and no others (sand lance, skate *Leucoraja ocellata*, Atlantic cod *Gadus morhua*, and Pleuronectidae *Pseudopleuronectes americanus*). The samples from the Aquarium of Niagara, from grey seals with known diets, contained OTUs from all 9 expected prey species and no others (skate, haddock *Melanogrammus aeglefinus*, striped bass *Morone saxatilis*, monkfish *Lophius americanus*, capelin, Atlantic mackerel, Atlantic herring *Clupea harengus*, Pacific herring, and rainbow smelt).

Qualitative comparisons of PCR reactions with and without the use of a grey seal blocking primer were used to validate blocking primer function. Agarose gels illustrated that the brightness of the band using grey seal template DNA was diminished with the use of the blocking primer, whereas the brightness of the band using a mix of fish DNA as a template was qual-

itatively unaffected by the presence of the blocking primer. The grey seal blocking primer was thus effective at minimizing predator DNA amplification without negatively impacting amplification of target prey items. However, the blocking primer was unable to completely eliminate grey seal DNA amplification, and grey seal DNA was detected in 12 samples (FO = 16.2%, mean RRA = 3.68%).

Lastly, we included a duplicate of a wild sample to evaluate consistency. The original and the duplicate sample shared 5 of 6 prey items as determined by FO. The shared prey items were as follows, with a comparison of RRA values between the original and duplicate samples respectively: *Merluccius bilinearis* (silver hake; 68.44 vs. 69.51%), red hake (9.13 vs. 6.64%), Pleuronectidae spp. (6.79 vs. 7.40%), cod (5.96 vs. 4.54%), and *Zoarces americanus* (ocean pout; 3.25 vs. 11.34%). With the exception of ocean pout, the shared prey species by FO were highly consistent in RRA values. Heterogeneous distribution of prey DNA in a scat or differential PCR amplification may account for some variation in FO or RRA results. By FO, the duplicate sample did not detect the presence of sand lance in the diet. However, there were sand lance reads in the duplicate sample; this prey group was filtered out in the 1% proportional cutoff used for OTU trimming. Nonetheless, the duplicate and original sample reflected relatively consistent results for both FO and RRA. Overall, the controls provide support for accurate prey detection and consistency of prey detection across samples.

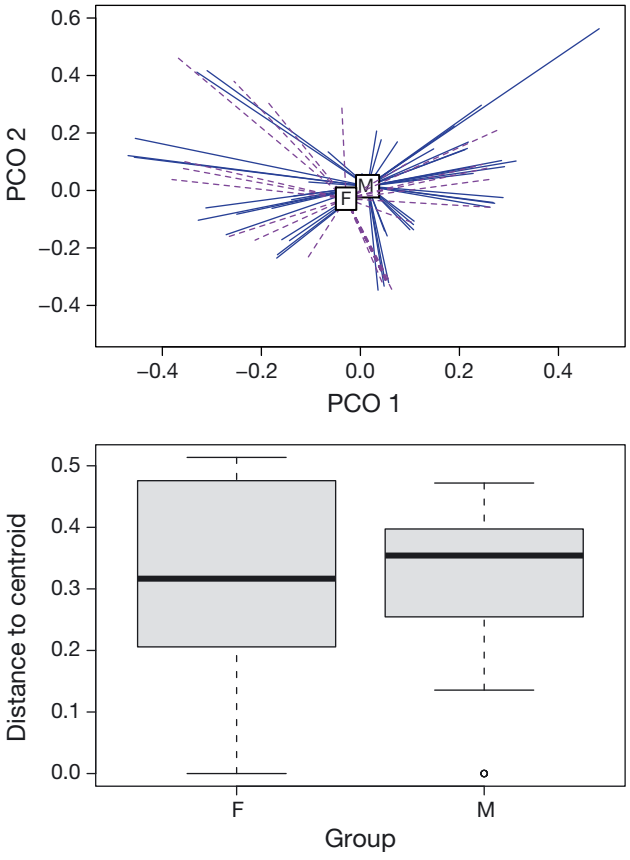

Fig. 5. Spider plot (top) depicting centroid locations and group dissimilarities based on prey presence/absence as calculated by 1 – Sørensen's index for males ('M'; blue solid line) and females ('F'; purple dashed line). Boxplots (bottom) illustrating the mean distance to the centroid for females ('F') and males ('M'). Shaded boxes: interquartile range; solid line within the shaded boxes: median value; whiskers: minimum and maximum values

3.4. Statistical analyses

Overall prey richness ranged from 1–8 within samples with an average (±1 SE) richness of 3.11 ± 0.18. Male samples contained all 24 prey groups while female samples contained 18 prey groups. There was no significant difference in prey richness between male (3.14 ± 0.19) and female (3.04 ± 0.39) grey seals by the Wilcoxon-Mann-Whitney test (W = 541, p = 0.49). Wilcoxon-Mann-Whitney tests also failed to detect any significant differences between RRA of any prey species in male and female samples (p > 0.05 for all comparisons).

Average dissimilarity between samples via the 1 – Sørensen index was 0.568 (range: 0–1). Sex did not significantly impact beta diversity as determined by 'adonis' ($F_{1,72}$ = 1.433, p = 0.25). Fig. 5 illustrates group dissimilarities between males and females as well as average dispersions from the centroid.

4. DISCUSSION

Molecular scatology is a valuable approach for studying foraging ecology in a variety of systems. In the case of New England grey seals *Halichoerus grypus*, detection of prey DNA in scat allowed for minimally invasive sample collection and accounted for digestion biases that affect traditional hard parts analysis. Pinniped diet analyses are often based on identification to species of prey hard parts present in scat; however, hard parts may be absent when seals fail to ingest otoliths or when otoliths degrade substantially or entirely (Jobling & Breiby 1986). Prey DNA, in contrast, can be recovered from ingested soft parts, meaning molecular scatology may detect prey items where hard parts analysis cannot (Deagle et al. 2005, Dunshea 2009). In comparison to other

methods of inferring feeding habits, such as stable isotope analysis or fatty acid analysis, our molecular scatology approach also attained higher taxonomic resolution. Sampling units for molecular scatology investigations are more easily and inexpensively obtained from haul-out sites, with minimal disturbance to the animals or risk to the researchers.

Our study examined grey seal feeding habits with the highest degree of taxonomic resolution since Ampela (2009), one of few studies to investigate US grey seals. Ampela (2009) examined both hard parts in scat and stomach contents of grey seals from the same island as the present study (Monomoy Island). Ampela (2009) detected 27 and 24 vertebrate prey groups in scat and stomach analyses (respectively), comparable to the 24 vertebrate prey groups identified in this study (Table 1). In total, 14 prey groups were found to overlap between this study and the scat analysis in Ampela (2009), although FO values were considerably different. Nonetheless, sand lance *Ammodytes* spp. were the most frequently consumed group overall for both studies, highlighting the reliance of seals on sand lance. The ecosystem impacts of sand lance are widespread, as evidenced by the correlations between sand lance abundance and populations of predator fish (Robards et al. 1999), seabirds (Robards et al. 1999), and baleen whales (Víkingsson et al. 2015). In the Gulf of Maine area in particular, humpback whales *Megaptera novaeangliae* also rely primarily on sand lance and have modified foraging behavior in response to sand lance movement and abundance (Friedlaender et al. 2009). As important forage fish, dedicated studies of sand lance population dynamics could better inform trends in predation by grey seals and broader trophic impacts of seal foraging.

Overall, Ampela (2009) defined 'important' prey species as those contributing to the diet at 5% or higher in frequency, number, and/or weight. By this measure, 6 species in her study were important via FO: sand lance, winter flounder *Pseudopleuronectes americanus*, skates (Rajidae), red/white hake *Urophycis* spp., windowpane flounder *Scophthalmus aquosus*, and cusk eel (Ophidiidae). In contrast, there were 13 important species in our study using the same 5% FO threshold. Notable species in our study which differ from Ampela (2009) include Atlantic menhaden *Brevoortia tyrannus*, Atlantic mackerel *Scomber scombrus*, and northern sea robin *Prionotus carolinus*, which were the second, fourth, and fifth most consumed species overall, respectively. Menhaden and sea robin were entirely absent from Ampela (2009), and mackerel occurred at only 1.1% FO (compared to 22.97% in our study). Our finding of 13 frequently consumed prey species is an unusually high value, as seals have traditionally been found to rely heavily on a few species (Benoit & Bowen 1990, Prime & Hammond 1990, Bowen & Harrison 1994, 2006, Hammond et al. 1994). However, Gallo-Reynoso & Esperón-Rodríguez (2013) described plasticity in foraging behavior of Guadalupe fur seals *Arctocephalus townsendi*, which are otherwise highly specialist feeders. Declining availability of primary prey items is a reasonable incentive for niche breadth expansion through foraging behavior plasticity (Spencer et al. 2017). Future studies which examine the population dynamics of important seal prey species (i.e. sand lance, flatfish, hakes, etc.) and constraints on grey seal foraging plasticity may further inform when and how seal feeding patterns change. Maternal behavioral plasticity has been documented in grey seals breeding on North Rona (Twiss et al. 2012), and the authors note the potential for similar plasticity in foraging behavior. An understanding of the foraging capabilities of grey seals is crucial to evaluating the extent of predation impacts across trophic levels and the potential for grey seal populations to continue expanding (Byron & Morgan 2016).

Our use of prey DNA rather than hard parts may have contributed to differences in FO as well as differences in detected prey groups between our study and Ampela (2009). Hard parts of skates and silver hake *Merluccius bilinearis* were found in 24.5 and 2.0% of samples, respectively, in Ampela (2009), compared to of 2.70 and 18.92% by FO in our study. Detection rates and passage time of prey DNA differs compared to those of hard parts (Deagle et al. 2005), which may lead to differences in results and interpretation of feeding habits. Furthermore, Atlantic menhaden and sea robin were among those species not detected in Ampela (2009), which documented anecdotal evidence of grey seals consuming both species specifically without ingesting the head. Indeed, more than half of our samples (FO = 60.81%) contained menhaden DNA and approximately one-fifth (FO = 21.62%) contained sea robin, though no menhaden or sea robin otoliths were found in ongoing hard parts analyses of the same scats (K. Ono unpubl. data). It is possible menhaden were highly underrepresented in previous hard parts studies, if seals avoid consuming the head and thus the otoliths of some prey species. Fluctuating availability of menhaden is unlikely to have contributed to the disparate findings between Ampela (2009) and the present study, as abundance and biomass of adult menhaden

generally increased along the US east coast from 2000–2013 (Simpson et al. 2016). The success of various prey detection methods is valuable for commercial fishery management as exemplified by menhaden, which is the most important species by landing volume in the Atlantic coast fishery (NOAA Fisheries 2020).

Although we focused on FO for comparable analyses to Ampela (2009), our RRA results were notably consistent with our FO values. Sand lance and Atlantic menhaden were the most important prey groups by both measures (97.30 and 60.81% FO, 55.17 and 14.40% RRA, respectively). *Hippoglossina oblonga* (fourspot flounder) was also the least important species by both measures, at 1.34% FO and 0.02% RRA. Arriving at similar conclusions for most and least important species using either FO or RRA reinforces the reliability of our broad interpretations regarding grey seal ecology. Deagle et al. (2019) noted that FO and RRA are more likely to align when there are few prey groups in individual samples; in our case, the average number of prey groups per scat sample was relatively low at 3.11 ± 0.18, further supporting the accuracy of our results through both diet measures.

There are also disadvantages to evaluating only one diet metric. For example, prey consumed in small quantities may be overemphasized if only FO is examined, and RRA could overestimate importance of certain species from amplification bias (Deagle et al. 2019). Two representative prey groups which are particularly disparate between FO and RRA in our study are *Urophycis regia* (spotted codling) and *Clupea harengus* (Atlantic herring). Spotted codling and Atlantic herring were present in 13.51 and 12.16% of grey seal samples, respectively, while only occurring at <1% by RRA. Atlantic herring is a valuable species to the Northeast fishing industry, and as such the quantity of fish consumed needs to be considered while determining marine mammal predation impacts (Overholtz & Link 2007). Utilizing FO and RRA in combination will allow for a more accurate understanding of the role of grey seals in the Northeast Atlantic ecosystem.

In addition to describing the prey species and groups which we detected via DNA in seal scats, we also examined the role of consumer sex in grey seal diet. Interestingly, sex of the seal did not have a significant effect on alpha or beta diversity, though there is clear evidence of diet differences in the literature through other methods (Beck et al. 2005, 2007). We did find that females consumed 18 species total compared to 24 species in males, which is consistent with previous findings that female grey seal diets are less diverse than that of males (Beck et al. 2007). Beck et al. (2007) suggested that females tend to forage on higher quality prey; however, our results do not provide a clear trend given the lack of significant differences between male and female diet. Ampela (2009) found that silver hake in particular was consumed more frequency by males than by females according to stomach content analysis; in our study, silver hake was consumed equally by both sexes and was consumed with higher total frequency (18.92 compared to 2.00%) compared to Ampela (2009).

Female grey seals in Nova Scotia, Canada, are known to forage closer to shore than males, but males tend to forage in slightly deeper waters (Breed et al. 2006). Females may be expected to encounter and consume skate (among other benthic species) more frequently inshore, though males may encounter skates more often on the shelf floor. We did not detect a difference in consumption of skate or any other benthic species between males and females. Studies of diving and movement patterns in Cape Cod breeding colonies may better inform our results. A recent study (Moxley et al. 2017) began investigating the potential for using telemetry data, in part to understand foraging and haul-out behavior in US grey seals. Overall, our results of the effect of sex on the diet support the need for multiple approaches to understanding foraging ecology. It remains unclear if the lack of significant diet differences between males and females here should be attributed to the use of different methodologies, to the changes in grey seal ecology and behavior over the 10–15 yr since many of the foundational studies were published (Beck et al. 2005, 2007, Breed et al. 2006, Ampela 2009), or to our sample size. It is important to note that our sex subsamples (50 male and 24 female) were relatively small, particularly for females. Disentangling these effects is key to understanding grey seal food habits and therefore trophic impacts in the Northeast Atlantic.

Although the use of prey DNA in this study allowed for new insights of US grey seal foraging, there are methodological drawbacks to molecular analyses of predator scat. Inherent disadvantages of utilizing scat-derived DNA include the presence of PCR inhibitors and the difficulties associated with obtaining degraded DNA (Waits & Paetkau 2005). The method is particularly prone to contamination, which may introduce cross-contamination and inflated FO and RRA of rare taxa. Furthermore, processing of next-generation sequencing data and taxa assignments are not straightforward and can influ-

ence the results. For example, the primer matching threshold in initial cleaning steps and the sequence similarity threshold used in OTU picking can impact the resulting data set. Lastly, the presence of secondary prey items has also been known to influence PCR techniques with invertebrates (Sheppard et al. 2005), and this method is not able to distinguish between primary and secondary prey.

There are also conflicting results from studies documenting the degree to which PCR can accurately quantify the proportions of prey consumed (Deagle et al. 2019). There is evidence that the relative proportions of prey consumed can be accurately deduced from qPCR results with the use of correction factors (Deagle & Tollit 2007, Bowles et al. 2011). Sequencing via Ion Torrent, however, was less successful in reconstructing seal diet proportions despite the use of correction factors (Thomas et al. 2014). Ongoing research is continuing to refine the possibility of estimating diet proportionally via next-generation sequencing methods (Thomas et al. 2016). Recently, Deagle et al. (2019) argued for inclusion of RRA in prey DNA studies, as RRA may be more reflective of population diet despite the inconsistent ability to accurately quantify diet.

Marine mammals are, in many cases, central to shaping the structure of communities and trophic webs in which they are involved (Estes & Palmisano 1974, Rau et al. 1992, Estes et al. 1998). An understanding of marine mammal foraging ecology underlies the ability to study how predation impacts the ecosystem. In the context of human societies, knowledge of marine mammal foraging is also crucial to commercial fisheries management. This study utilized molecular techniques to provide novel information, with species-level resolution in most cases, about feeding habits of grey seals in New England. Positive controls from both a tissue-extracted fish mix sample and samples from captive seals with known diets suggested that our study successfully captured fish prey contents of wild grey seal scat samples through FO, and we report RRA as a proxy for diet quantification to be interpreted with knowledge of the caveats. Hard parts analyses of the samples involved in this study are ongoing, but comparison to a previous study (Ampela 2009) indicates that our next-generation sequencing approach identified instances of prey items that hard parts have failed to detect (e.g. menhaden, sea robin). Both of these species were in the top 5 prey groups consumed as determined by FO, so it is important that they are not underestimated in analyses of various grey seal foraging impacts in New England. However, our study also generally agrees with hard parts findings that indicate seals are primarily consuming sand lance, which are important forage fish. We demonstrated through the use of prey DNA that traditional hard parts analysis does not appear to underestimate grey seal consumption of highly valued fish species such as Atlantic cod *Gadus morhua* or salmon *Salmo salar*. Despite this, we also found that niche breadth of grey seals may be larger than previously understood (given the high number of important species) or may have expanded, and future studies should continue to monitor the relationship between seals and Gulf of Maine fishes. Improved knowledge of pinniped diet will, in turn, better inform our ability to study the trophic web and societal impacts associated with pinniped populations in coastal communities.

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