# ASSESSMENT OF TUFTED PUFFIN *FRATERCULA CIRRHATA* DIET USING DNA METABARCODING

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**ABSTRACT**

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Investigating trophic relationships can be critical for understanding relationships between marine predators and their prey. DNA analysis of feces is used increasingly as a non-invasive method to uncover seabird dietary patterns across space and time. Tufted Puffins *Fratercula cirrhata* are listed as Endangered in the state of Washington (WA), USA, and reduced prey availability is thought to be a key factor in the species' decline. Recent information on Tufted Puffin diet is lacking, and present opportunities for direct diet observation are limited. We conducted a pilot study to characterize Tufted Puffin diet on Destruction Island, WA, in 2019 using DNA metabarcoding of feces from burrow entrances and from soil in nesting chambers. Smelt (Osmeridae) and rockfish (Scorpaenidae) were detected in all fecal samples, along with a variety of other fish taxa, squid, crab, and shrimp. Smelt was detected in most soil samples, as were a variety of other fish, crustaceans, and terrestrial insects. While DNA metabarcoding detected several taxa also identified in Tufted Puffin bill-loads in 2019, fecal and soil samples detected multiple taxa not identified in bill-loads. It appears that Tufted Puffin diet can be characterized using DNA metabarcoding, provided that fecal samples are of sufficient quality and that contamination is minimized. Amplifying prey DNA from soil samples opens opportunities for sampling burrows after breeding, which would minimize disruptions to study colonies. Future strategies to characterize Tufted Puffin diet could combine direct observation and DNA metabarcoding methods where possible and could focus on the latter methods where observation is difficult. These non-destructive and non-disruptive methods hold promise for characterizing the diet of other burrow-nesting species of conservation concern.

**Key words:** metabarcoding, fecal DNA, Tufted Puffin, California Current

## INTRODUCTION

Trophic connections between seabird species and their prey are often gleaned from observational studies that are conducted at one or a few locations or that do not sample throughout the breeding season. This can result in an incomplete picture of seabird diet and limit our understanding of both seasonal changes in food resource availability and the effects of perturbations to prey in a changing ocean environment. For some consumer species (i.e., species of conservation concern), data may be limited and/or logistically challenging to collect, and the problem can be even more acute. These limitations make it clear that we need to use a variety of sampling methodologies.

Tufted Puffins *Fratercula cirrhata* range throughout the temperate and sub-arctic North Pacific, with large colonies in the Aleutian Islands and along the Alaskan Peninsula. They also breed in significant numbers in southeastern Alaska (USA) and British Columbia (Canada), and in lower numbers in Washington, Oregon, and California (USA; Piatt & Kitaysky 2020). Population declines over the last century led the state of Washington to list the species as Endangered in 2015 (Hanson *et al.* 2019). While Tufted Puffin is not listed under the US Endangered Species Act (USFWS 2020), further population declines predicted in the coming decades threaten the species' persistence in the California Current Large Marine Ecosystem (LME; Hart *et al.* 2018). Negative trends have been documented at Tufted Puffin colonies in the California Current and Gulf of Alaska LMEs, which together represent 75% of the species' North American range (Pearson *et al.* 2023).

Of the factors identified for the Tufted Puffin's decline in the California Current LME, reductions in prey abundance and/or timing of prey availability appear to be important (Hanson & Wiles 2015). Forage fish availability, which is a function of oceanographic factors and fishery depletion, influences seabird foraging behavior and eventual breeding success. In British Columbia, variation in reproductive performance of Tufted Puffins has been associated with sea-surface temperature (Gjerdrum *et al.* 2003), and Borstad *et al.* (2011) found that prey availability was likely related to variable timing and intensity of primary production. The consequences of

shifts in prey composition and/or distribution can be extreme in some cases. Food web changes during marine heat waves have led to high seabird mortality dominated by Tufted Puffins (Jones *et al.* 2019).

A range of species has been documented as Tufted Puffin prey in Alaska and British Columbia by Hanson & Wiles (2015). Fish provisioned to chicks include Pacific Sandlance *Ammodytes hexapterus*, Pacific Herring *Clupea pallasii*, juvenile rockfish *Sebastes* spp., Eulachon *Thaleichthys pacificus*, Pacific Sardine *Sardinops sagax*, Northern Anchovy *Engraulis mordax*, Walleye Pollock *Theragra chalcogramma*, greenlings *Hexagrammos* spp., and Capelin *Mallotus villosus*, along with squids, euphausiids, and some other invertebrates. Tufted Puffin diet has been investigated at the Farallon Islands off the California coast (Ainley & Boekelheide 1990), but little is known about diet elsewhere in the California Current (Hanson & Wiles 2015).

Breeding Tufted Puffins are particularly sensitive to colony disturbances. Assessment of Tufted Puffin diet on the outer Washington coast has relied on photographic documentation of provisioning adults returning to the colony and motion-activated trail cameras deployed next to burrow entrances. While these direct methods reduce researcher interactions and provide diet information (including prey size), they can suffer from poor taxonomic resolution, misidentification, and bias against small prey. Moreover, employing these methods on multiple islands is logistically complicated and involves extensive investments of time, energy and money.

Organisms shed genetic material into the environment; this is called environmental DNA or eDNA. Over the last decade, strides have been made in extracting and sequencing eDNA from a variety of environmental media (water, soil, feces, etc.) to identify the organisms from which it came (Thomsen & Willerslev 2015). One method is DNA metabarcoding, which allows the identification of multiple prey species in mixed samples such as animal excretions and stomach contents. It is an emerging non-invasive method for determining the diets of marine vertebrates that has been used on tunas (Trujillo-González *et al.* 2022), sea turtles (Díaz-Abad *et al.* 2022), and marine mammals (Ford *et al.* 2016, Thomas *et al.* 2017, Michaux *et al.* 2021). Results from eDNA studies can often be correlated with results from studies using conventional sampling methods (Port *et al.* 2016, Thomsen *et al.* 2016, Kelly *et al.* 2017, Sigsgaard *et al.* 2017, Pont *et al.* 2018), most of which involve greater time and energy investment. One limitation of eDNA methods is that secondary prey are difficult to distinguish from primary prey.

For seabirds, diets have been assessed using DNA analysis of feces from several species, including penguins (Deagle *et al.* 2007, Jarman *et al.* 2013, Cavallo *et al.* 2018), albatrosses (McInnes *et al.* 2016a, 2017a, 2017b), shearwaters (Komura *et al.* 2018, Nimz *et al.* 2022), terns (Bogantes *et al.* 2024), and cormorants (Oehm *et al.* 2017). The eDNA methods perform as well as or better than conventional dietary characterization methods for many species (Deagle *et al.* 2007; Bowser *et al.* 2013; Jarman *et al.* 2013; McInnes *et al.* 2017a, 2017b; Cavallo *et al.* 2018), but not for all (Oehm *et al.* 2017).

Here, we present exploratory work to demonstrate the potential for using dietary DNA metabarcoding to characterize Tufted Puffin diet. We assessed fecal and soil samples from Tufted Puffin burrows using Illumina sequencers, which provided large amounts of high-quality sequence data that allowed us to characterize the species composition of many samples in parallel. In addition, we investigated nanopore sequencing, which provided long DNA sequence reads that allowed for the exclusion of unwanted sequences. Finally, we compared our results with diet characterizations based on direct observations.

## STUDY AREA AND METHODS

### Field fecal samples

Fecal samples were collected in July and August 2019 from a Tufted Puffin colony on Destruction Island (47.6749°N, 124.4839°W), a 12 ha (0.12 km$^2$) island located 6 km off the Washington coast (Fig. 1). We collected fresh or dried fecal material from the entrances of known Tufted Puffin burrows using sterile wooden stirrers, placed them in labeled Eppendorf centrifuge tubes containing at least 2.5 mL of 95% ethanol, and shook the tubes to mix and preserve the contents.

### Field soil samples

Soil samples were collected in August 2019 from the same Tufted Puffin colony on Destruction Island. We collected soil from nesting chambers by scooping the upper 2-cm layer using a sterile 50-mL plastic centrifuge tube grasped in a 60” flexible, four-claw pick-up tool. We guided the tool-held tube down the burrow tunnel to the nesting chamber using an infrared camera system (Peep-A-Roo, Sandpiper Technologies; Manteca, California) that we use to monitor puffin egg and chick development. This system is composed of a camera and infrared diodes (LEDs) in a plastic head at the end of a 3-m rubber-coated stainless flex-tube, video-display goggles, and a battery pack. We transferred soil samples from the collecting tubes to labeled Eppendorf centrifuge tubes containing at least 2.5 mL of 95% ethanol and shook the tubes to mix and preserve the contents.

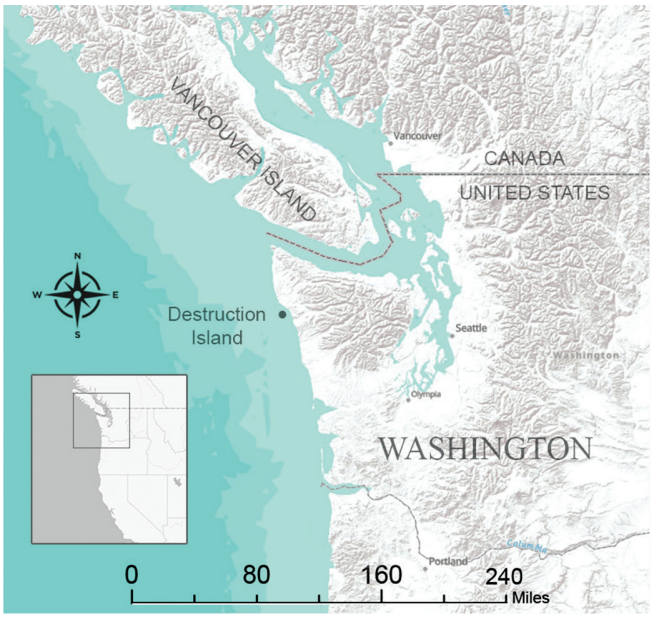


**Fig. 1.** Map showing location of Tufted Puffin *Fratercula cirrhata* colony on Destruction Island, Washington, USA, in the northern portion of the California Current Large Marine Ecosystem.

**Zoo fecal samples**

To develop and optimize the molecular methods, fecal samples were collected from July to September 2019 from Tufted Puffins housed in the Rocky Shores habitat at the Point Defiance Zoo and Aquarium in Tacoma, Washington. When individuals were observed defecating, fecal samples were immediately scraped from the surface of the exhibit with sterile wooden stirrers, being careful not to touch the surface with the stick. Samples were placed in labeled Eppendorf centrifuge tubes containing at least 2.5 mL of 95% ethanol and shaken to mix and preserve the contents. Samples were transported to the lab and stored covered at room temperature until they were prepared for DNA extraction. Samples of prey fed to the puffins around the time of fecal sample collection were obtained from the zoo; these included one individual each of herring (unknown species), Capelin, Atlantic Silverside *Menidia menidia*, and Antarctic Krill *Euphausia superba*.

**DNA extraction and amplification**

Fecal ($n$ = 9) and soil samples ($n$ = 8) from the field and fecal samples from the zoo ($n$ = 9) were extracted using a QIAamp Fast DNA Stool Mini Kit (catalog no. 51604, Qiagen; Hilden, Germany) and eluted in 100 µL. Tissue samples from the four prey items from the zoo were extracted using a DNeasy Blood & Tissue Kit (69504, Qiagen).

The island and the zoo exhibit both host several marine bird species, so all samples were tested for species origin by amplifying (via polymerase chain reaction, PCR) and Sanger sequencing a 125-base pair (bp) fragment using the primers Aves-16S-1AF and Aves-16S-1AR from Dalén *et al.* (2017). Resulting amplicons were sequenced on an ABI 3500 Genetic Analyzer (Applied Biosystems, Inc.), and samples identified as Tufted Puffin were taken through the next steps.

To identify the prey component of samples, we used fish primers (16S1F-Ext and 16S2R, Deagle *et al.* 2007) and crustacean primers (Mala16S-R and 16S-inv-R, Fountain *et al.* 2023) on prey tissue samples provided by the zoo before using them on fecal and soil samples. Primer details are in the Appendix (available on the website). These two primer sets were used to maximize the capture of diversity across taxa. To prevent origin (host) species amplification, the puffin blocker TuftedPuffin-16S-Rblock (based on Bowser *et al.* 2013) was used. PCR amplification details are in the Appendix. In addition to the non-template (negative) controls, the same primer pairs were used on DNA extractions of known species to act as positive controls. Successful PCR products of the prey component were purified and individually indexed. The resulting amplicons were purified again and quantified before pooling them. The pooled library was metabarcoded using a MiSeq Reagent Kit v3 (600 cycle; catalog no. MS-102-3003, Illumina; San Diego, California) on a MiSeq benchtop sequencing system (Illumina). For details, see the Appendix.

**Metabarcoding sequence bioinformatics**

DNA sequences were demultiplexed to their sample of origin by lllumina's proprietary sequencing software. The resulting paired reads were processed using a pipeline that combines third-party software (Gallego 2021) to split the dataset by PCR primers and to generate amplicon sequence variants (ASVs) for each sample/primer combination. ASVs were further checked for chimeras using the *vsearch* tool (Rognes *et al.* 2016) and clustered into operational taxonomic units (OTUs) with the Swarm program (v.2, Mahé *et al.* 2015). Individual OTUs were assigned a taxonomical identity using the Basic Local Alignment Search Tool (BLAST) hosted by the National Center for Biotechnology Information (Maryland, USA). For each query sequence, up to 50 matches that returned over 85% similarity were recovered. From those matches, a final taxonomic identity was assigned by calculating the last common ancestor using the function *condenseTaxa* from the R package "taxonomizr".

**Nanopore sequencing**

The DNA samples remaining from the previous analysis ($n$ = 25) were used in a pilot study to determine the length of the prey DNA and the long-fragment metabarcoding capability using the MinION Mk1B sequencing platform with adaptive sampling (Oxford Nanopore Technologies or ONT; Alameda, California). Eleven subsamples (from two fecal and five soil samples from the field and one fecal sample from the zoo) produced visible bands. Each sample was cleaned individually, washed twice with 70% ethanol, and eluted in 12 µL of nuclease-free water. Samples were pooled equimolarly, and a library containing a total mass of 720 ng was loaded on a R10.3 flow cell (FLO-MIN111, ONT), following the manufacturer's instructions. The sequencing run was set up to enrich the prey and exclude the Tufted Puffin signal for the adaptive sampling setting on the MinKnow sequencing system (ONT). The raw output (voltage signal) was translated to a DNA sequence using the software program *Guppy 4.3.4* and the super accurate (SUP) basecalling model (ONT). Individual samples were demultiplexed using the native barcodes, clusters of similar sequences were delimited using Decona v.1.3.1 (Oosterbroek *et al.* 2021), and the number of sequences associated with each sample and cluster was obtained with a custom script. The taxonomic identification of the clusters' consensus sequences was obtained in the same way as with the Illumina-generated sequences.

**RESULTS**

**Verification of avian DNA via Sanger sequencing**

For the field fecal samples, avian DNA was amplified in six of the nine samples collected from burrow entrances. Tufted Puffin DNA was detected in all six samples; however, two of the samples also contained relatively large amounts of DNA from gulls (Laridae, Fig. 2A). Avian DNA was amplified in six of the eight field soil samples collected from Tufted Puffin nesting chambers. In all six samples, Tufted Puffin DNA was detected; a small amount of gull DNA was detected in one of those samples (Fig. 3A). For the zoo samples, avian DNA was amplified in all four fecal samples tested. Two samples contained Tufted Puffin DNA, while the DNA in the two other samples was assigned only to a higher taxonomic level (family Alcidae); one of the samples with Tufted Puffin DNA also contained DNA from Common Murre *Uria aalge*, another alcid housed in the Rocky Shores habitat.

**Prey identification via metabarcoding**

We obtained more than 14 million (M) reads belonging to 130 unique barcode combinations: 124 samples and six positive controls. Field and zoo samples together totaled 13.56 M reads, of which 98% (13.40 M) passed quality filters. These were denoised by *dada2* (Callahan *et al*. 2016) independently in the forward

and reverse directions, and 12.28 M reads were successfully merged. We searched for chimeric sequences and kept 11.61 M reads from 773 ASVs that cleared those filters, which were later clustered into 139 OTUs using Swarm. The average read depth was 94 000 sequences per unique barcode combination.

Prey DNA of fishes, cephalopods, and crustaceans was amplified in three of the four fecal samples from the field that were verified as Tufted Puffin samples. One sample (Field Fecal-1) had very few ASVs, none of which were assigned to prey species. While a fair amount of ASVs could not be resolved lower than "ray-finned fish," several samples included ASVs matching sequences at the family, genus, and species level. Smelt (Osmeridae) and rockfish (Scorpaenidae, *Sebastes* sp.) were detected in all three samples; a right-eyed flounder (Pleuronectidae) and Showy Snailfish *Liparis pulchellus* were detected in two samples; Wolf Eel *Anarrhichthys ocellatus*, Speckled Sanddab *Citharichthys stigmaeus*, a herring *Clupea* sp. (likely Pacific Herring), a cod (Gadidae), and Quillfish *Ptilichthys goodei* were detected in one sample each (Fig. 2B). For cephalopods, an armhook squid *Gonatus* sp. and a pencil squid (Loliginidae) were detected in one sample each (Fig. 2B). For crustaceans, a commensal crab (Pinnotheridae) was detected in two samples, while copepods (*Calanus pacificus* and *Pseudocalanus elongatus*) and a shrimp *Pandalus* sp. were detected in one sample each (Fig. 2B).

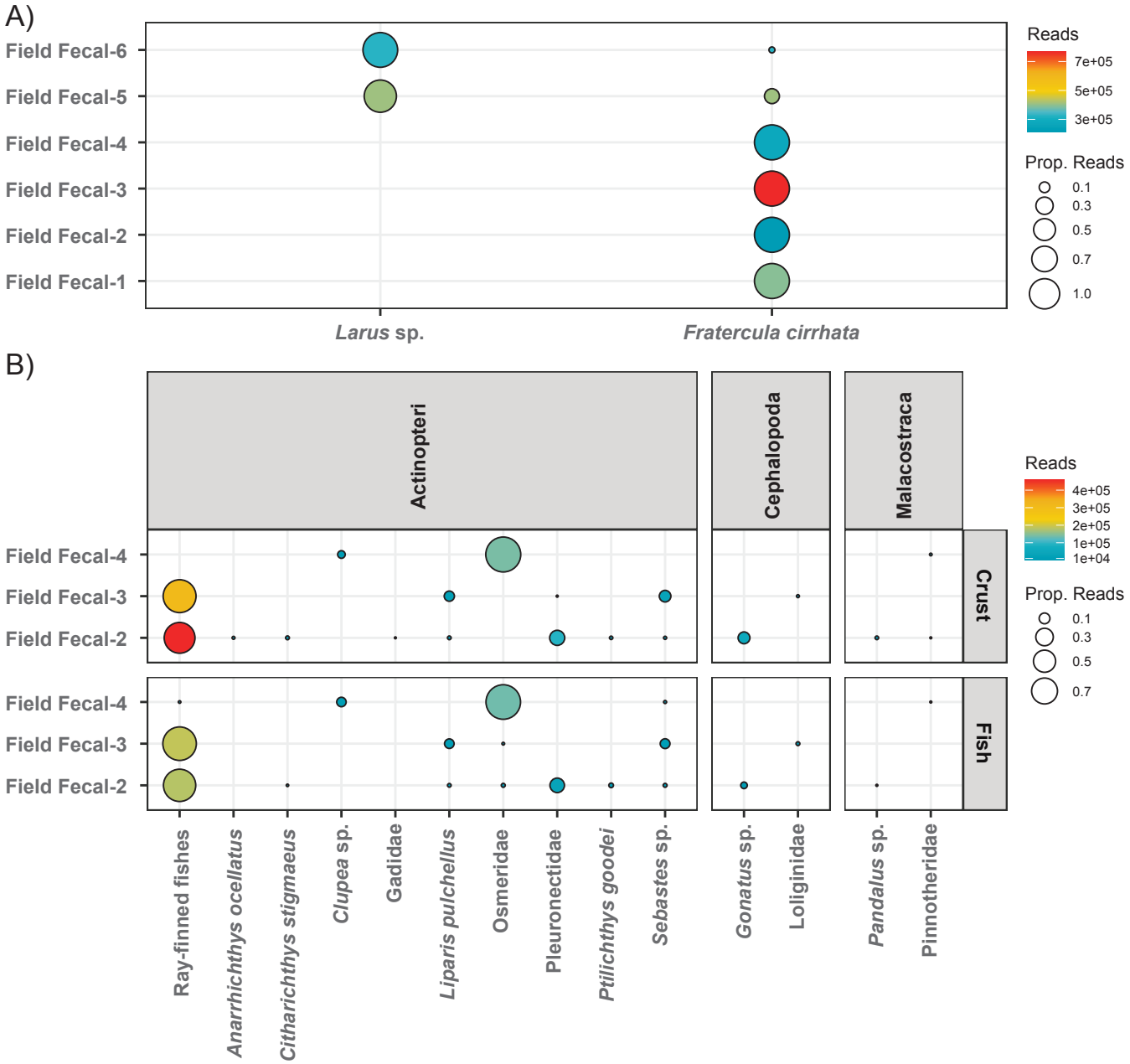


**Fig. 2.** Composition of fecal samples collected from burrow entrances at the Tufted Puffin *Fratercula cirrhata* colony on Destruction Island, Washington, USA, as derived from metabarcoding analysis: (A) number and proportion of amplicon sequence variants of avian DNA identified from fecal samples using the amplicons resulting from 16S AVES primers, and (B) number (Reads) and proportion (Prop. Reads) of amplicon reads (among putative prey species) using 16S crustacean (Crust) and fish (Fish) primers.

Prey DNA of fishes and crustaceans was amplified in five of the six soil samples from the field that were verified as from Tufted Puffins; one sample (Field Soil-1) had no prey ASVs. For fish, smelts were detected in four samples, while Pacific Saury *Cololabis saira*, a herring *Clupea* sp. (likely Pacific Herring), a hake *Merluccius* sp. (likely Pacific Hake *M. productus*), a Pacific salmon *Oncorhynchus* sp., rockfish, Night Smelt *Spirinchus starksi*, and Longfin Smelt *S. thaleichthys* were detected in one sample each (Fig. 3B). Arachnids, Slender Springtails *Homidia anhuiensis*, a springtail (Entomobryidae), and a barklouse *Liposcelis* sp. were detected in single samples each (Fig. 3B). An unresolved malacostracan crustacean was detected in one sample (Fig. 3B). The DNA signal strength (as indexed by read abundance) for the soil samples was weak relative to the zoo and field fecal samples, except for one soil sample (Field Soil-5). There were slight differences between results using different primers: hake and rockfish were detected by only the fish primer set, and terrestrial invertebrates were detected by only the crustacean primer set.

Prey DNA of fishes and crustaceans was amplified in the four samples from the zoo. Prey included herring, Capelin, Atlantic Silverside, and Antarctic Krill. A stickleback (Gasterosteidae) and Rainbow Smelt *Osmerus mordax* were detected in two samples, although these taxa were not part of the zoo-provided puffin diet. The number and proportion of DNA sequence reads in samples (denoted by color warmth and circle size) show that Capelin was the dominant prey DNA detected in the samples, followed by Atlantic Silverside, herring, and Antarctic Krill (Fig. 4).

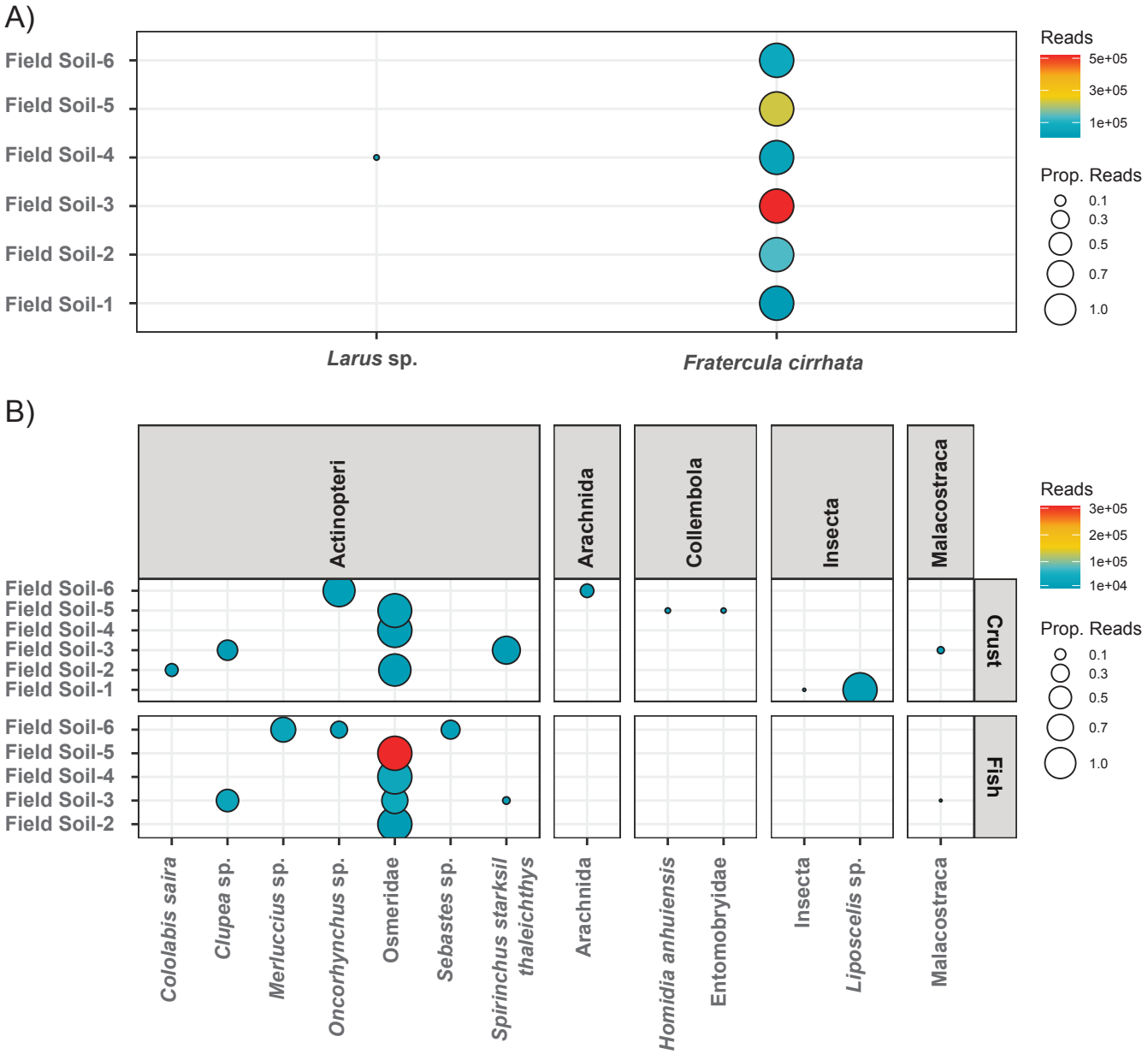


**Fig. 3.** Composition of soil samples collected from burrow chambers at the Tufted Puffin *Fratercula cirrhata* colony on Destruction Island, Washington, USA, as derived from metabarcoding analysis: (A) number and proportion of amplicon reads of avian DNA identified from fecal samples using the amplicons resulting from 16S AVES primers; (B) number (Reads) and proportion (Prop. Reads) of amplicon reads (among putative prey species) using 16S crustacean (Crust) and fish (Fish) primers.

### Nanopore sequencing

Only 44% of the remaining DNA samples amplified for the long fragment (almost 2000 bp), which spans from mid-12S rRNA to almost-complete 16S rRNA genes. Of the long reads, 80% (70% of the ASVs) matched *Homo sapiens* (99% pairwise identity), while the remaining sequences belonged to two fish species. Despite the use of adaptive sampling or selective sequencing to reject DNA of Tufted Puffin origin from the nanopores, sequences of up to 1200 bp were found. These originated from at least three Tufted Puffin individuals, since three different haplotypes of the 16S rRNA gene were found (see Appendix). In the fecal samples collected from the zoo, Atlantic Silverside was detected with 99.95% pairwise identity, which matched the prey sample provided in the zoo. The only other species found was Fourline Snakeblenny *Eumesogrammus praecisus* (97.33%), which was detected in three field samples (two soil and one fecal) originating most likely from three different Tufted Puffin individuals, given their three different 16S haplotypes.

## DISCUSSION

### Characterizing diet using DNA metabarcoding

We successfully extracted avian and putative prey DNA from most samples processed (six of nine fecal samples, six of eight soil samples, four of four zoo samples). All zoo samples and nearly all field fecal and soil samples were from Tufted Puffins; two field fecal samples that were not from puffins were likely from members of the Glaucous-winged Gull *Larus glaucescens*/Western Gull *L. occidentalis* hybrid complex that breed on the island and are commonly observed in and around the puffin breeding colony. As Tufted Puffins at this colony are skittish, we restricted sampling to brief visits into the colony following prey observation bouts (two in July and one in August). This constraint on trips, along with the small colony size (*ca.* 20–25 active burrows), limited the number of samples we could collect. This limit contrasts with the hundreds of samples that can be collected easily by working in dense seabird colonies (Jarman *et al.* 2013; McInnes *et al.* 2017a, 2017b; Cavallo *et al.* 2018) or by capturing individual birds (Bowser *et al.* 2013, Fountain *et al.* 2023).

Fecal sample analysis indicated that Tufted Puffins at this colony prey on fish and invertebrates; metabarcoding identified forage species (smelts, herring) as well as some benthic species (snailfish, Wolf Eel) not found in puffin diets in the California Current. Potential gadids include Pacific Cod *Gadus macrocephalus*, which are known puffin prey along the Alaska Peninsula (Piatt & Kitaysky 2020), and Pacific Tomcod *Microgadus proximus*. Snailfish are a deepwater, sandy-bottom fish and seem to be unlikely prey, but their juveniles have been observed in the diet of Atlantic Puffins *Fratercula arctica* (Bowser *et al.* 2013). Wolf Eels are deepwater, benthic predators, but their pelagic juveniles may also fall prey to puffins. Small numbers of flatfish and other bottom fish observed in diets elsewhere have fueled speculation that Tufted Puffins also forage in benthic habitats (Piatt & Kitaysky 2020). Invertebrate taxa identified included squid and shrimp, which are likely prey of appropriate size that have been detected in diets elsewhere in the California Current. Prey species of lower trophic levels are often detected in Tufted Puffin diets, especially earlier in the breeding season (Williams *et al.* 2008, Davies *et al.* 2009). Other taxa include commensal crabs, which are more likely secondary prey of Tufted Puffins via planktivorous fishes (Bowser *et al.* 2013).

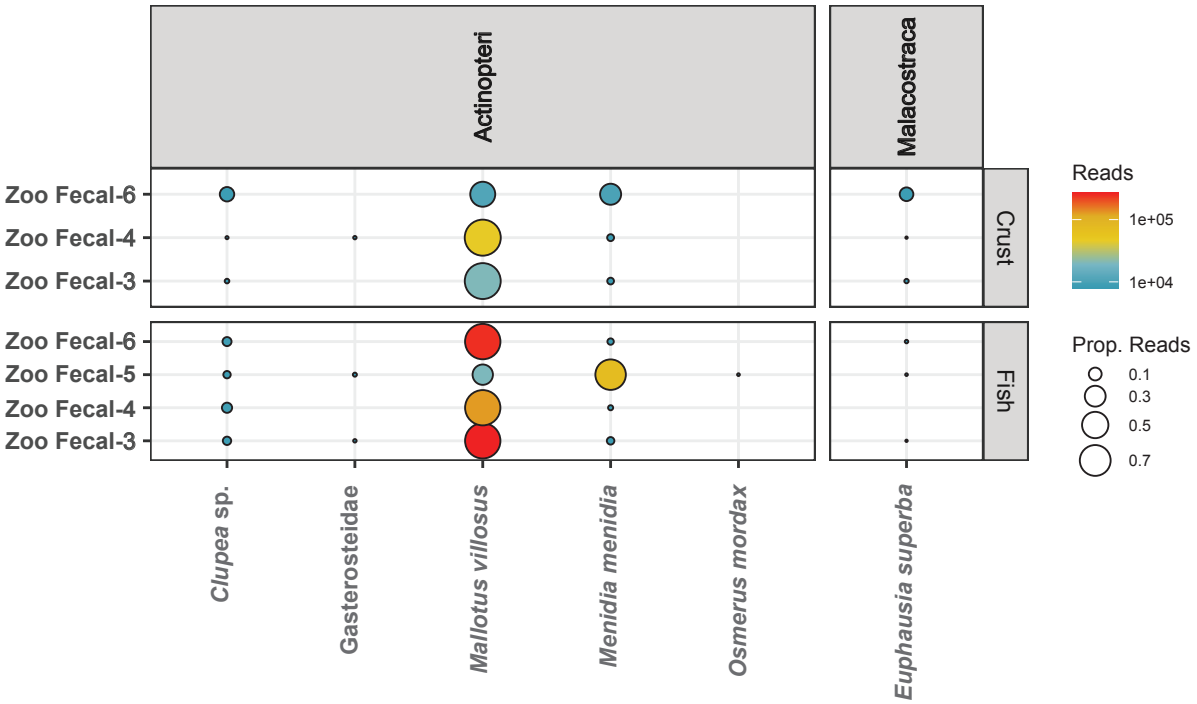


**Fig. 4.** Composition of Tufted Puffin *Fratercula cirrhata* fecal samples collected from the Rocky Shores habitat at the Point Defiance Zoo and Aquarium in Tacoma, Washington, USA, showing number and proportion of amplicon reads of number (Reads) and proportion (Prop. Reads) of amplicon reads among putative prey species using 16S crustacean (Crust) and fish (Fish) primers. All samples contained Tufted Puffin DNA.

Soil analysis also characterized the Tufted Puffin diet at this colony as consisting mainly of fish. As with the fecal samples, metabarcoding identified California Current species (Pacific Saury, smelts, herring) as well as taxa unknown as Tufted Puffin prey in the California Current, such as hake and salmonids. Invertebrate taxa identified (arachnids, springtails, etc.) are common terrestrial or shoreline taxa, and they are unlikely to be Tufted Puffin prey along Washington's outer coast. These invertebrates were not detected in our fecal samples, and springtails have been known to contaminate fecal samples before collection by researchers in penguin colonies (Jarman *et al.* 2013).

Our metabarcoding analyses generally agree with what is known about Tufted Puffin diet elsewhere in the North Pacific. Pacific Sandlance and Pacific Herring rank as important prey in Alaska and British Columbia. In addition, juvenile rockfish, Eulachon, Pacific Sardine, Northern Anchovy, Walleye Pollock, Greenling, Capelin, squids, euphausiids, and some other invertebrates have been documented (Hanson & Wiles 2015). At Triangle Island, British Columbia, and in Chiniak Bay, Alaska, adult Tufted Puffins shift their diet over the course of the nesting season. Early in the season, they forage on abundant pelagic zooplankton and squid of modest quality, and they switch to coastal fish species of higher nutritional quality later in the season when they need to feed growing chicks and keep themselves fortified for long provisioning flights (Williams *et al.* 2008, Davies *et al.* 2009). In the waters of central California during summer, Northern Anchovy, juvenile rockfish, and Market Squid *Doryteuthis opalescens* dominated the diet fed to chicks, with anchovy prevalence decreasing over time from 1973 to 1982 (Ainley & Boekelheide 1990).

**DNA metabarcoding vs. photo identification**

While both DNA-metabarcoding and photo-identification methods documented smelts, herring, and squid, metabarcoding analyses revealed a broader Tufted Puffin diet. Taxa identified from only metabarcoding fecal samples included gadids, flatfish, rockfish, snailfish, wolffish, and shrimp, while taxa identified from only metabarcoding soil samples included hake, salmonids, rockfish, and saury. Pacific Sandlance was the lone taxon documented from photo identification only (SFP unpubl. data). In 2019, much of the prey observed in Tufted Puffin bill-loads was unidentified larval fish (~73%), so some of these larval fish may have been identified in fecal and soil samples. Larval fish were less common from 2016–2022 (0%–48% of bill-loads); in those years, only flatfish was added through photo identification.

Some taxa not photo-identified in Tufted Puffin bill-loads have been documented in bill-loads collected from the closely related Rhinoceros Auklet *Cerorhinca monocerata* at Destruction Island (Wagner *et al.* 2024). In 2019, the diet of Rhinoceros Auklet was found to include some taxa that were identified in metabarcoding of Tufted Puffin fecal and soil samples, including salmonids, flatfish, rockfish, and squid species; in other years, Rhinoceros Auklet diet has also included Pacific Tomcod and Pacific Saury (TPG unpubl. data). Surprisingly, Northern Anchovy was not documented in Tufted Puffin diet at this colony through metabarcoding or photo identification. Anchovy is an important forage species in the California Current, especially in central California waters, where it contributes 57%–94% by mass of the Tufted Puffin diet, depending on year (Ainley & Boekelheide 1990, Weber *et al.* 2021). Northern Anchovy has decreased in Rhinoceros Auklet diet on Destruction Island from > 50% in 2008 to ~9% in 2016 and thereafter (TPG unpubl. data). Thus, our metabarcoding data may reflect the decrease in availability and/or use already underway of this forage species in waters around Destruction Island.

Characterizing a broader diet using DNA methods is common in studies comparing methods of determining diet. Fecal DNA analyses have revealed higher diversity of prey compared with stomach sampling for Macaroni Penguin *Eudyptes chrysolophus* (Deagle *et al.* 2007), Adelie Penguin *Pygoscelis adeliae* (Jarman *et al.* 2013), and Little Penguin *Eudyptula minor* (Cavallo *et al.* 2018). The molecular approach outperformed morphological hard-part identification regarding the detectable prey spectrum and prey species composition for pellets, feces, and regurgitated fish samples of Great Cormorants *Phalacrocorax carbo sinensis* (Oehm *et al.* 2017). For Atlantic Puffins, the number and diversity of taxa identified using DNA metabarcoding was greater than conventional diet assessment from observation blinds; some taxa—Bluefish *Pomatomus saltatrix* and Rock Gunnel *Pholis gunnellus*—were identified only through DNA analyses (Bowser *et al.* 2013). Metabarcoding analysis can produce results more taxonomically resolved than conventional diet assessment, which may not be able to identify prey to the species level. The contrast between results can be especially stark, as it was for our study. Identifying prey in Tufted Puffin bill-loads on Destruction Island through photography is especially challenging, as provisioning adults circle the colony any number of times, land unexpectedly, and enter burrows quickly.

**Caveats and methodological points**

General caveats with respect to DNA metabarcoding results involve sample quality, sample contamination, interpretation of results as primary vs. secondary prey, and gaps in the DNA catalog that underlies taxon identification. DNA metabarcoding also cannot provide information on prey size/age, both of which are important in assessing energetic aspects of diet. Fecal samples collected from burrow entrances were exposed to the elements, no doubt resulting in some sample degradation. Humidity, exposure time, and temperature all promote DNA degradation, even in the absence of rain and direct sunlight (Naef *et al.* 2023), and genomic DNA extracted from fecal samples can be of poor quality from inherent enzymatic and bacterial digestion (Bowser *et al.* 2013). We did not experience ideal field conditions for collecting avian feces for DNA analysis (ideal: fresh samples collected from smooth, clean, non-absorbing surfaces that are protected from sunlight and rain; Oehm *et al.* 2011, McInnes *et al.* 2016a). Still, DNA was by and large successfully extracted from even dried-out fecal samples.

Despite our best efforts, samples collected in both field and zoo settings were contaminated by other species. Designing and employing a human DNA blocker was critical to getting usable results. Contamination from gull fecal material was effectively controlled by screening for avian DNA source; however, minimizing the potential for swamping prey DNA signals is likely more important when collecting samples from the colony surface than when collecting feces directly from birds captured on the water (Fountain *et al.* 2023). Even in the zoo setting, samples linked to observed defecation events of known individuals could have been contaminated by other alcid species present in the exhibit (Common Murre, Horned Puffin *Fratercula corniculata*), reinforcing the idea that screening for avian DNA source is prudent. The zoo presented

unique contamination issues, as taxa not reported as puffin food by zoo personnel (stickleback and smelt) were identified in fecal samples. These taxa were likely mixed in with the fish that the zoo buys to feed puffins and other birds in the exhibit.

Characterizing diet from DNA metabarcoding can be fraught, as it is challenging to interpret whether ASVs come from primary prey versus secondary prey consumption. However, we can often make plausible inferences about whether some taxa represent secondary prey. Some Tufted Puffin prey are themselves planktivorous (herring, smelts, saury); therefore, some ASVs may have emanated from the planktivores' prey. While Tufted Puffins can accumulate several prey items in their bills on foraging trips (Piatt & Kitaysky 2020), it is unlikely that they amass non-target taxa much smaller than their gape, such as copepods and planktonic invertebrate stages. Moreover, stomach samples of Pacific Herring and Pacific Sandlance captured by Rhinoceros Auklets at Destruction Island are dominated by copepods, amphipods, and decapods (M. Galbraith unpubl. data). In ~20% of Atlantic Puffin fecal samples, metabarcoding identified crustaceans, copepods, and cladocerans. These taxa were considered unlikely target prey of puffins, and they were documented in stomachs of herring dropped by provisioning puffins (Bowser *et al.* 2013). Seabird diet studies using molecular methods often attribute invertebrate ASVs to secondary predation, accidental ingestion during foraging, or prey parasites. Support for these inferences comes from conventional stomach-content sampling (Nimz *et al.* 2022) and measuring diagnostic bones (Thalinger *et al.* 2022), but these techniques were not available to us in this study.

In some instances, metabarcoding results could not be resolved to the species or genus level (e.g., maximum resolution of "Actinopteri"). This has often been attributed to taxa not being previously sequenced at the loci of interest, deficiencies in the reference database (GenBank), sequencing error (Bowser *et al.* 2013), or lack of resolution at a given locus. A deeper dive into the database, which we did not do, may not result in any further prey identification. Uncertainty about the expected taxonomic diversity can be addressed by employing multiple group-specific primers, but that approach still may not uncover the taxonomic range of the prey consumed (Bowser *et al.* 2013). Our primers revealed overlapping but not identical sets of taxa, so using multiple primers was justified; however, approaches for combining data from multiple eDNA markers to infer diet compositions are in their infancy and likely require additional statistical complexity.

We had hoped that the zoo setting would afford an opportunity to align DNA signal strength from fecal samples with consumption of prey by captive puffins, but that was not possible. During daylight hours, individual feedings were documented, as captive birds were hand-fed. However, during the overnight period, all the birds in the exhibit were provided with a large tray of mixed prey, so it was not possible to document prey intake of individual birds. Determining the relationship between strength of DNA signal from prey taxa could be explored empirically in follow-up studies using mock communities of a known mix of species and analyzing the corresponding ASV output (see Shelton *et al.* 2023). For these reasons and more, we refrained from interpreting the strength of the signals as representing dietary proportions.

The preliminary results from the nanopore sequencing suggest that the length of the fragment was beyond the integrity of the diet DNA. A multimarker metabarcoding approach might help identify more species, particularly if using barcoding genes such as 12S and 16S rRNA. The adaptive sampling approach will not work as well with shorter fragments, given the high-speed sequencing of the ONT technology, but it could be implemented to reject as much endogenous DNA as possible. Sequencing of short amplicons with greater than 99% accuracy is currently feasible on ONT platforms. Hence, future ONT sequencing would benefit from a multimarker sequencing approach of shorter fragments (e.g., 12S, 16S, *cyt* b, COI). This approach could maximize the chances of amplifying DNA from the diet, while the adaptive sampling method with puffin and human DNA could screen out contamination, particularly from field samples. The identification of Fourline Snakeblenny in two samples was surprising, as this fish is found in the northern Arctic and the Bering Sea (Froese & Pauly 2023). It may have come from a local related species, such as one of the pricklebacks and shannies (family Stichaeidae); even so, these small, benthic fish are not known to be Tufted Puffin prey. Gunnels (family Pholidae) are a similarly small, benthic species that were an unexpected taxon identified in chick fecal samples in Atlantic Puffins (Bowser *et al.* 2013). It could also be an artifact from the combined 12S and 16S rRNA genes being sequenced here that resulted in a fragment sequence that is underrepresented in the public databases.

**Potential future work**

Environmental DNA techniques consistently show promise in describing diet and identifying predator-prey relationships, despite recognized limitations and often challenging circumstances. Future characterization of the Tufted Puffin diet in the California Current is more likely to happen through the inclusion of these techniques. Non-invasive molecular methods can provide high-level taxonomic prey resolution and can verify diet more effectively than conventional sampling methods, which consume more time and energy (Port *et al.* 2016, Thomsen *et al.* 2016, Kelly *et al.* 2017, Sigsgaard *et al.* 2017, Pont *et al.* 2018). Results can reveal previously unknown patterns in prey selection, foraging behavior, and ecosystem linkages. Many studies have revealed unknown reliance on deepwater fish (Komura *et al.* 2018, Carreiro *et al.* 2021) or gelatinous taxa (Jarman *et al.* 2013, Cavallo *et al.* 2018). Still others revealed that non-breeders consume different prey groups compared to breeders, possibly reflecting differential foraging ranges, selectivity, or foraging experience (McInnes *et al.* 2016b). Such insights could be especially helpful for the Tufted Puffin in places where it is a rare and difficult-to-study species of conservation concern.

In the northern part of its range in Alaska, Tufted Puffin adults reportedly rely on invertebrates for their own sustenance while their chicks are provisioned with fish (Piatt & Kitaysky 2020). To characterize the diet of both Tufted Puffin adults and chicks in the California Current, metabarcoding fecal and soil samples from multiple colonies could be combined with photo-documentation where conditions allow. Tufted Puffin adults tend to defecate outside the nest chamber, whereas chicks defecate in the nest chamber when small and at the burrow entrance when older (Piatt & Kitaysky 2020). Thus, fecal samples from burrow entrances during egg brooding and the early chick period could capture adult diet, while photographic sampling of provisioning adults and nest-chamber soil samples could capture chick diet. Fecal DNA analysis of Atlantic Puffin adults and chicks in the northwestern Atlantic revealed that

their diets were indistinguishable from each other (Bowser *et al.* 2013); this assumption is untested due to constraints at breeding colonies, where seabird diet studies often focus on chick diet.

Adding other sampling methods to metabarcoding efforts could provide insights into Tufted Puffin diet and foraging. Sampling the stomachs of fish prey can help differentiate primary from secondary consumption in metabarcoding results, as results from fecal and soil samples considered in isolation from fish diet could lead to erroneous conclusions about the foraging biology and diet of puffins (Bowser *et al.* 2013). Although Tufted Puffin prey are difficult to obtain on Destruction Island, easy access to Rhinoceros Auklet prey could facilitate stomach sampling. Where feasible, data loggers could be attached to provisioning Tufted Puffin adults, which can provide additional insights into foraging ecology (Komura *et al.* 2018). Capturing individuals at the colony or on the water would facilitate fecal sample collection. Using a combination of DNA metabarcoding techniques, data loggers, and camera traps, Fayet *et al.* (2021) showed that Atlantic Puffin adults experienced poor productivity when foraging further afield due to low prey availability near their colonies. Fecal DNA metabarcoding combined with metrics recorded using Passive Integrated Transponder tags and platform scales (i.e., individual foraging-trip duration and changes in body mass) could also document seasonal and annual shifts in prey choice as well as potential changes in ecosystem conditions (Cavallo *et al.* 2020). For Tufted Puffin colonies where observations and photo-documentation are challenging, sporadic visits to collect fecal and soil samples in combination with these other techniques could increase the spatial extent of sampling throughout the California Current, where populations have been in decline (Pearson *et al.* 2023).

The metabarcoding results from the nesting-chamber soil samples are particularly promising moving forward, as soil sampling after the breeding season could establish burrow occupancy as well as characterize diet while avoiding disturbance. Nest chambers are fairly deep (1–2 m) and relatively removed from heat and light, so puffin and prey DNA is less likely to degrade (Naef *et al.* 2023). DNA in soil samples from burrows has been used to confirm occupancy by terrestrial reptiles (Nordstrom *et al.* 2022), just as DNA from scat and feather samples has been used to verify the recolonization of Macquarie Island in Tasmania by burrow-nesting petrels (McInnes *et al.* 2021). For Tufted Puffins, soil sampling could verify breeding on islands where field operations are difficult to conduct but nesting chambers are accessible. Soil samples from burrow chambers likely integrate DNA deposited on the chamber floor over longer time periods than DNA incorporated in fecal samples; thus, soil samples could be collected before Tufted Puffin breeders arrive, during their breeding, and then after chicks fledge to examine temporal patterns of DNA signals.

At the best of times, conventional methods of characterizing predator diets can suffer from poor taxonomic resolution, misidentification, and bias against small or completely digestible prey. DNA-based barcoding techniques have become a powerful tool for diet reconstruction from stomach contents or fecal samples, either for comparative purposes with previously known diets or for *de novo* diet description (Bowser *et al.* 2013). As a non-invasive method, recovering DNA of prey species from the environment has emerged as a method of determining diet in a variety of taxa (including marine birds) and in a variety of conservation and environmental settings, such as that which currently exists for Tufted Puffins along the west coast of the USA. The use of DNA metabarcoding on fecal and soil samples has the potential to substantially increase our understanding of the diet and foraging ecology of Tufted Puffins, a species of conservation concern. These methods also exemplify the promise of molecular techniques to increase the quality and quantity of studies examining predator-prey relationships in the face of constraints posed by traditional data-collection techniques.

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