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An inside "beak": Molecular analysis of swab samples reveals the seabird diet of invasive Barn Owls in Hawai'i

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Abstract

Predation is an important species interaction to monitor when assessing an invasive species' impact on a particular ecosystem, but it can be difficult to observe and thus, fully understand. On Kaua'i island, invasive Barn Owls (*Tyto alba*) predate native seabirds, but difficult terrain in this region and the cryptic nature of owl predation make traditional monitoring of predation quite challenging. Using Barn Owls collected as part of removal efforts on Kaua'i and Lehua islands, we conducted DNA metabarcoding of owl digestive tracts to detect and determine seabird species they predate. We used a seabird-targeted *12s* marker to sequence 112 swabs from 55 owls and detected 6 seabird taxa, including 2 ESA-listed seabirds—Hawaiian Petrel (*Pterodroma sandwichensis*) and Newell's Shearwater (*Puffinus newelli*), in 12 swabs from 11 owls (20% of sampled owls). Corresponding morphological assessment of owl stomach contents detected seabird species as prey items in only 2% (1/55) of sampled owls, highlighting the utility of molecular approaches for detecting diet items, especially degraded or visually absent items. Additionally, this approach has proven very useful in revealing cryptic trophic interactions in inaccessible seabird populations. For the most comprehensive analysis of diet, the use of both esophageal and cloacal swabs for metabarcoding is recommended. Supplementing metabarcoding with other methods that can provide complementary prey information, such as stable isotope analysis, would help to characterize trophic interactions more fully. The method described here has proven to be a reliable tool for investigating diet in invasive owls and may be used to investigate cryptic predation in living birds as a minimally invasive technique, as well.

Graphical Abstract

Key words: invasive species, cryptic predation, DNA metabarcoding, Tyto alba

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Introduction

Ever since the seminal paper "Facts from feces revisited" by Kohn and Wayne was published in 1997 ecologists have realized the power of molecular biology to help gain new insights into animal ecology. Five years earlier, Hoss et al. (1992) used PCR to analyze brown bear feces from the Brenta region of Italy and found Christmas berry, *Photinia villosa*, fruits dominating the late summer diet. This was an item often missed with traditional fecal diet analysis (Putman 1984). Putman's (1984) original "Facts from feces" recognized that traditional diet analysis would be limited because 1) plant and animal fragments may be differentially recognized and counted in feces, and 2) these dietary materials would be digested and passed through the gut at different rates causing bias in feeding behavior studies. However, it was not until Kohn and Wayne (1997) that the broader ecological community realized the power of using molecular approaches to evaluate animal feces to estimate population sizes of the target species, evaluate home range and territory size, assess effective population size, measure genetic variation, evaluate diet, and gain new insights into unseen interactions between predators and prey.

Molecular diet assessments have the potential to unveil trophic interactions that are difficult to observe, particularly when the predator is secretive and no morphological evidence of predation is left behind (e.g. Kohn and Wayne 1997; McInnes et al. 2017). The application of molecular tools to these questions has been a recent area of innovation and growth in conservation genetics (Wayne and Morin 2004; Boyi et al. 2022; Pilgrim et al. 2023). With the advent of high throughput sequencing tools, metabarcoding—parallel sequencing of taxonomically informative amplicons from pooled samples—has become a particularly powerful molecular tool with application to a wide range of wildlife research studies including wildlife forensics, foraging behavior, population monitoring, and habits of invasive species (Staats et al. 2016; Lin et al. 2021; Kryshak et al 2022; Mas-Carrio et al 2022; Roffler et al. 2023; Tosa et al. 2023). Newer studies have applied metabarcoding tools to a variety of sample sources beyond feces including, intestinal contents, carrion flies, and cloacal swabs (Young et al. 2020; Massey et al. 2021; van Zinnicq Bergmann et al 2021; Corriveau et al. 2022; Fernandes et al. 2023).

One area of research which may especially benefit from the application of diet metabarcoding is cryptic predation by invasive species. Invasive predators have the potential to impact the survival of native fauna and reduce biodiversity, particularly in island ecosystems (Reaser et al. 2007; Bellard et al. 2014; Keppel et al. 2014, Mayfield et al. 2021). Invasive predators are recognized as one of the largest threats to native ecosystems and are one of the leading causes behind species endangerment (Mayfield et al. 2021). In fact, invasive predators have been linked to the extinction or endangerment of 738 vertebrate species and have contributed to more than 50% of all bird, mammal, and reptile extinctions (Savidge 1987; Rogers et al. 2017; Doherty et al. 2016).

Barn Owls (*Tyto alba*) were introduced to the Hawaiian island Kaua'i in 1959 as a rodent biocontrol agent, but they have had unintended predatory impacts on native seabirds with the first demonstrated unintended effects being shown as early as 1980 via direct observation of predation or evaluation of prey remains (Tomich 1962; Au and Swedberg 1966; Byrd and Telfer 1980). Since their introduction, Barn Owl populations have grown rapidly throughout the main Hawaiian Islands, due in part to their ability to produce multiple clutches per year with large brood sizes (del Hoyo et al. 1999). The ability of Barn Owls to adapt diet preferences has led to seabird predation of multiple species in Hawai'i (Raine et al. 2019, 2021). Kaua'i island and the neighboring islet Lehua are home to some of these native seabird species including the endangered 'ua'u (Hawaiian Petrel, *Pterodroma sandwichensis*), the Threatened 'a'o (Newell's Shearwater, *Puffinus newelli*), the Endangered 'akē'akē (Band-rumped storm-petrel, *Oceanodroma castro*), 'ā (Red-Footed and Brown Booby, *Sula sula* and *Sula leucogaster*), 'ou (Bulwer's Petrel, *Bulweria bulwerii*), noio (Hawaiian Black Noddy, *Anous minutus*), and 'ua'u kani (Wedge-tailed Shearwater, *Ardenna pacifica*). In addition to Barn Owls, other seabird predators introduced to the Hawaiian Islands include rodents (*Rattus rattus*, *R. exulans*, *R. norvegicus*, and *Mus musculus*), cats (*Felis catus*), and feral swine (*Sus scrofa*), all of which can impact seabird biodiversity and species survival through the depredation of eggs, chicks, and adults (Seto and Conant 1996; Hu et al. 2001; Smith et al. 2002; Raine et al. 2020). Although the impacts of rodents, cats, and feral swine on Hawaiian seabirds are well documented—facilitated by the relative ease of live trapping and observing these individuals at nest sites—the impacts of Barn Owls are less well understood (Caut et al. 2008; Towns et al. 2011; Harper and Bunbury 2015).

Many of the seabird colonies on Kaua'i that Barn Owls predate upon are restricted to the northwestern rainforests and Na Pali coast where dense vegetation and steep terrain make seabird colony monitoring difficult. Evidence of Barn Owl predation is found infrequently, but often enough to initiate regular Barn Owl control in these areas (Ainley et al. 2001; Raine et al. 2017b). When evidence is found, it is usually at nest sites in the form of depredated seabirds that have been beheaded and stripped of muscle and viscera—injuries distinctive to owl predation (Raine et al. 2021). On Lehua islet, where there is limited vegetation and moderately steep terrain, evidence of Barn Owl predation of native seabirds is found more frequently (VanderWerf et al. 2007). It may be assumed that similar rates of predation occur on Kaua'i but remain unobserved due to site inaccessibility. Revealing the incidence of these cryptic predation events is important because without an understanding of the scope and relative intensity of trophic interactions, it is not possible to accurately predict how management actions to remove these top trophic predators may impact the broader ecological system.

Typical diet studies on Barn Owls rely on pellets as the source of diet samples, but Barn Owls appear to largely limit consumption of larger seabirds to muscle tissue and may have no hard parts in their digestive system or egested pellets for morphological species identification (Yom-Tov and Wool 1997; Raine et al 2019; Carlile and Lloyd 2022). Additionally, traditional monitoring of seabird nest sites may miss depredation events by owls, as owls frequently remove their prey from the burrow to eat elsewhere and predation events on seabirds sometimes occurs mid-air away from the colony (Khalafalla and Iudica 2012; Raine et al. 2017a, 2019). Thus, the full extent of this invasive species impact is difficult to assess using traditional sampling tools. In this study, we use molecular metabarcoding of Barn Owl cloacal and esophageal

swabs to detect predation of seabirds on Kaua'i and Lehua islands. By doing so, we aim to provide insight into the extent of seabird predation by Barn Owls on Kaua'i and illustrate the utility of molecular approaches for revealing cryptic trophic interactions in inaccessible seabird populations.

Methods

Sample collection

We sampled owls that were collected as part of predator removal programs conducted by Hallux Ecosystem Restoration (US Fish and Wildlife Service Migratory Bird Treaty Act Depredation permit number PER0046142; Hawai'i Department of Land and Natural Resources—Natural Area Reserve permit numbers I1265, I1307, and I2471; Kaua'i National Wildlife Refuge Complex permits 12519-19-048 and 12530-19-060; and Hawai'i State Parks Permit K2019-4073cc), the Kaua'i Department of Land and Natural Resources Department of Forestry and Wildlife, and National Tropical Botanical Garden predator control staff. Removal efforts were conducted in proximity to threatened and endangered seabird colonies known to contain *P. sandwichensis*, *Puffinus auricularis newelli*, *O. castro*, *S. sula*, *S. leucogaster*, *B. bulwerii*, *A. minutus*, and/or *A. pacifica*. The invasive species lethal removal methods used here were developed by Kyle Pias and Alex Dutcher (K. Pias and A. Dutcher, personal communication). Barn Owls were attracted to owl elimination sites using both recordings of Barn Owl territorial and "kleak" calls and a Barn Owl decoy located 15 to 30 m from the hunters. Owls were euthanized using a shotgun and non-lead ammunition. Owls were hunted between the hours of sunset and sunrise year-round, in compliance with local laws and regulations on these lands. Two additional owls were collected as roadkill by Kaua'i community members, relying on prior knowledge that road-killed animals have proven to be a reliable source of prey DNA in other molecular diet studies (Lee et al. 2013; Drake et al. 2023). Barn Owl carcasses sampled were collected between August 2017 through September 2022 (n = 55; Fig. 1). Twenty carcasses were sampled for prey genetic material immediately upon return from collection in the field (Piaggio et al. 2020) and the other 35 carcasses were stored at (−17 °C) until time of sample collection (Frantzen et al. 1998; Oehm et al. 2011). Frozen carcasses were allowed to thaw at room temperature for 30 min prior to sample collection. All owls were also necropsied and had stomach contents visually assessed for comparison with molecular analysis.

We sampled the cloaca and esophagus of each animal by swabbing with cotton-tipped applicators, an approach yielding better results than other sample types (see below). The cloaca and esophagus samples were sampled with separate swabs. For both cloaca and esophagus swabs the cotton applicator was inserted such that the cotton tip was no longer visible, then rotated five times before being placed in an individual sterile vial which was then labeled and frozen. Two individuals had duplicate esophageal and cloacal swabs collected, two individuals had only esophageal swabs, and the remaining 51 individuals had esophageal and cloacal swabs collected (n = 112 total swabs from 55 total animals). Swabs were then shipped overnight on ice to the National Genomics Center for Wildlife and Fish Conservation (Missoula, Montana, USA) where they were stored at −20 °C prior to DNA extraction. We assumed swabs had the greatest potential to collect DNA from prey consumed by owls within the last 8 to 12 h, as that is presumably when food items would be actively digesting (Barton 1992). Although it is possible prey DNA remains in the digestive system for hours, perhaps days, after it is consumed, the probability of detecting this DNA likely starts to significantly decrease some point following digestion (Schattanek et al. 2021). This swab sampling design (based on Peelle et al. 2019) was based on a pilot study to determine the best sampling medium for detecting seabird DNA from depredated owls. In that pilot, we isolated DNA from digestive tract contents (n = 31 animals; Appendix A) as well as swabs from cloaca and esophagus (n = 2 total swabs from one animal). The sample size for swabs is much smaller compared with digestive tract samples because the latter was expected to be more successful. However, sequencing efforts on digestive tract contents returned only owl DNA, while both swab samples returned owl and seabird DNA. This is likely because swab samples contained lower proportions of owl DNA, which allowed for less abundant seabird prey DNA to be amplified and sequenced.

DNA extraction

We extracted DNA from swabs using the Qiagen DNeasy Blood and Tissue kit (Qiagen Inc., Valencia, California) using the manufacturer's protocol with the following modifications: Swabs were incubated overnight at 55 °C in 400 µL ATL and 60 µL Pro-K. After sample digestion, 400 µL AL was added to the samples and thereafter, default instructions were followed. Extracted DNA was stored at −20 °C prior to analysis. Cloacal and esophageal swabs were processed separately for the entire analysis. Sample extraction was done in a laboratory space dedicated to low quality, noninvasive samples where PCR products and other sources of high-quality DNA are not handled.

Primer set modification, PCR, and sequencing

We amplified extracted DNA at a locus within the mitochondrial *12S* gene (200 bp). To target potential seabird prey species and reduce amplification of predator DNA, we modified and used the MiBird primers, a universal primer set designed for birds (Ushio et al. 2018; Table 1). To do so, we first performed an alignment with the original MiBird primers and the sequences of the *12S* gene for Barn Owl as well as 17 seabird species (Supplementary Table 1) using MEGA version X (Kumar et al. 2018; Stecher et al. 2020). The original MiBird primers would not bias against Barn Owl, so we screened the *12S* sequences for a region that was unique to the seabird species, but also had enough variation between seabirds for species differentiation. Within this region, we identified a SNP that is unique to the seabird species and not shared by Barn Owl. We designed a new forward primer that would incorporate this SNP as the last nucleotide on the 3′ end, as a locked nucleic acid. We did not have to modify the original MiBird reverse primer for the newly selected region. These primers are appended with Illumina adapter sequences for one-step adapter ligation and indexing. Samples were amplified in triplicate to avoid PCR drop-out with PCR volumes of 12 µL containing 2.6 µL of sterile H_2O, 6 µL 2× KAPA HiFi HotStart ReadyMix (Roche), 0.7 µL of each primer (5 µM), and 2 µL template, as in Ushio et al. (2018). We used a touchdown PCR profile to further reduce nontarget amplification and primer dimer formation.

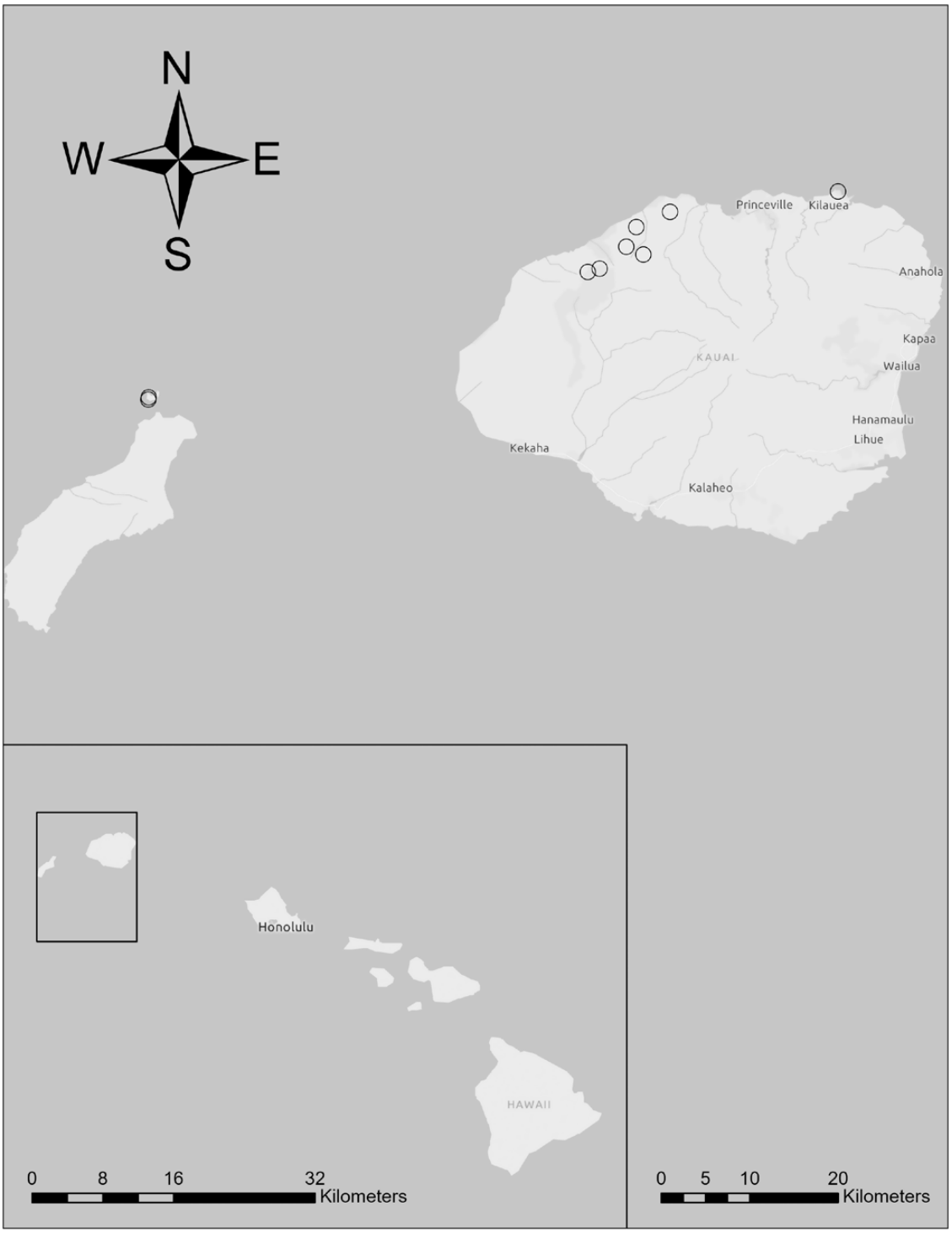

Fig. 1. Collection sites for the 11 Barn Owls with seabird diet detections on Kaua'i and Lehua islands. Two owls were collected on Lehua and nine were collected on Kaua'i (some owls were collected from the same location on Kaua'i; Samples 858 and 1072 and samples 918 and 1401).

Thermocycling conditions were as follows: 95 °C/3 min, [98 °C/20 s, 70 °C/15 s (−1 °C/cycle), 72 °C/15 s] × 10 cycles, [98 °C/20 s, 60 °C/15 s, 72 °C/15 s] × 30 cycles, 72 °C/5 min. Our control library was extracted DNA from Greater Sage-Grouse (*Centrocercus urophasianus*) tissue. The purpose of this library was 2-fold: as Greater Sage-Grouse is not present in Hawai'i, its presence in any libraries other than its own would be indicative of contamination during library prep, therefore, it can act as a control to track contamination. Additionally, our primer set amplifies Greater Sage-Grouse DNA, so it also acts as a positive control to monitor PCR and thermocycling conditions.

The PCR products were purified with AmPure SPRI beads at 0.9× and eluted in 20 µL EBT (10 mM Tris-Cl) to remove dimers and size select the desired amplicon (Beckman Coulter Inc. Pasadena, California). We electrophoresed each

PCR reaction on an agarose gel to validate whether target amplicons were produced and to assess DNA quality. We then pooled purified PCR triplicate reactions (10 µL/triplicate) for indexing, generating one cloacal swab library and one esophageal swab library per individual (with the exception of two owls with just esophageal swabs and two owls with duplicate swabs). We indexed these pooled products in amplification volumes of 25 µL containing 5 µL of sterile H_2O, 12.5 µL 2× KAPA HiFi HotStart ReadyMix, 1.25 µL of each indexing primer (10 µM), and 5 µL template (Table 1). The PCR profile was 98 °C/30 s, [98 °C/10 s, 60 °C/20 s, 72 °C/20 s] × 6 cycles, 72 °C/10 min. To reduce the risk of index swapping, each P5 and P7 index was used only once during indexing PCR. We used a fluorometer (Qubit 2.0; Life Technologies, Broad Range Assay kit) to determine DNA concentration of each library and pool libraries equimolarly at 150 ng to generate equivalent sequencing depth for all samples. A final bead clean with AmPure SPRI beads at 0.9× was performed and DNA was eluted into 20 µL EBT. The pooled libraries were sequenced separately on an Illumina MiSeq using the 300 Micro V2 kit to generate paired-end 150 bp reads (University of Montana Genomics Core).

Data processing and analysis

Sequencing reads were demultiplexed with Illumina software, *bcl2fastq*, and processed through a bioinformatic pipeline based around the *DADA2* package (Callahan et al. 2016) in *R* (R Core Team 2023). First, we used *cutadapt* (Martin 2011) to remove primer and adapter sequences. We then used the *DADA2* package function *filterAndTrim* to remove sequences with any ambiguous bases or more than two expected errors based on quality score estimates, to truncate sequences at the first instance of a quality score ≤4, and to discard any resulting sequences <25 bp. We trained the *DADA2* sequencing error rate model on our data using pooled sample data for each MiSeq run, then used the output to infer the original template sequences (i.e. amplicon sequence variants [ASVs]), and remove chimeric sequences. Reverse reads were of consistently low quality (all reverse reads mean per base sequence quality = 28.64), so forward and reverse reads were not merged and analysis was based on forward reads only. Filtered sequences were summarized in an ASV table and taxonomically assigned using a BLAST (Altschul et al. 1990) of the NCBI GenBank database and local alignment to newly generated reference sequences (www.ncbi.nlm.nih.gov/blast; Accessed March 2023). Species-level assignments were made if a BLAST hit had a 100% query coverage and at least 97% identity with the ASV; otherwise, a higher-level taxonomic assignment (family or genus level) was made (Leray et al. 2013). We employed a "Max Contamination" method for setting detection thresholds and considered any library with a seabird ASV read count higher than that same ASV within our Sage-Grouse "control" to be a detection (Drake et al. 2022). We chose this filtering approach as it has been shown to effectively minimize false positives while reducing the risk of removing true positives with low read counts (Lance et al. 2022). This may be especially pertinent for swab samples, which may generate ASVs with low read counts due to low initial DNA concentrations (Miles et al. 2015; Hanssen et al. 2017).

Supplemental Sanger sequencing

Complete *12S* reference sequences were not available for two high-priority seabird species: *P. sandwichensis* and *P. auricularis newelli*. We used Sanger sequencing to generate *12S* reference sequences for these species plus *A. pacifica* to supplement data available on GenBank (Table 1). We extracted DNA from the feathers of each species (collected from shed feathers or deceased animals; no sampling permits required) with Qiagen DNeasy Blood and Tissue kit (Qiagen Inc., Valencia, California) using the manufacturers protocol with the following modifications: The blood spot in the rachis was removed from the rest of the feather, placed in a tube with 300 µL ATL and 20 µL DTT (400 mg/mL) and Pro-K, and incubated at 55 °C overnight. The rest of the extraction steps followed the manufacturers' protocol for tissue samples. Samples were then amplified in volumes of 30 µL containing 13 µL of sterile H_2O, 3 µL 10× reaction buffer (Life Technologies; New York, USA), 2.5 mM $MgCl_2$, 200 µM each dNTP, 4 µL each primer (10 µM), and 1 U AmpliTaq Gold polymerase (Life Technologies). The PCR profile was 95 °C/10 min, [94 °C/1 min, 55 °C/1 min, 72 °C/1 min 30 s] × 34 cycles, 72 °C/5 min. The quality and quantity of PCR products were assessed with 1.6% agarose gel electrophoresis. Two amplicons of differing sizes were generated, with the smaller amplicon expected to be a *12S*

Table 1. PCR and sequencing primers used. The primer region for all primers is *12S* rRNA. Italics indicate MiSeq sequencing primers, bold X's indicate unique indices for multiplexing, and underlined characters represent Illumina P5/P7 adapter sequences. The nucleotide labeled "T+" in MiBird-U-F_alt_ tailed indicates a locked nucleic acid used to bias against *Tyto alba* amplification

Primer name	Primer type	Sequence (5′ to 3′)	References
MiBird-U-F_alt	PCR	*ACACTCTTTCCCTACACGACGCTCTTCCGATCT*CGGTCAC ACAAGARACCCAAAT+	Ushio et al. (2018)
MiBird-U-R	PCR	*GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT*CATAG TGGGGTATCTAATCCCAGTTTG	Ushio et al. (2018)
2nd PCR-F	PCR	AATGATACGGCGACCACCGAGATCTACAC **XXXXXXXX** *AC ACTCTTTCCCTACACGACGCTCTTCCGATCT*	Illumina Inc., San Diego, California
2nd PCR-R	PCR	CAAGCAGAAGACGGCATACGAGAT **XXXXXXXX***GTGACT GGAGTTCAGACGTGTGCTCTTCCGATCT*	Illumina Inc., San Diego, California
L1263	Sequencing	YAAAGCATGRCACTGAA	Sorenson et al. (1999)
H2294	Sequencing	TYTCAGGYGTARGCTGARTGCTT	Sorenson et al. (1999)

nuclear mitochondrial DNA segment (nuMT). For certainty, we performed a gel extraction using the QIAquick Gel Extraction Kit (Qiagen Inc., Valencia, California) on both amplicons and sequenced them separately. To remove excess primers and nucleotides, 4 µL of ExoSAP-IT, 4 µL of PCR product, and 6 µL of sterile H_2O were combined and incubated as follows: [37 °C/15 min, 80 °C/15 min] × 1 cycle. Purified PCR products and primers (10 µM) were shipped to Eurofins on dry ice and sequenced (2 reads/sample) using the PlateSeq service (Eurofins USA, Luxembourg City, Luxembourg). We trimmed sequences, removed low-quality bases, and merged reads into the final sequence assemblies (~971 bp long each) using Sequencher (version 5.4.6, Gene Codes Corporation). The final *12S* sequences are available on GenBank (accession numbers: OR388121, OR388122, and OR388123).

Results

Sequencing of swab samples

Sequencing of 55 cloacal libraries and 57 esophageal libraries generated 811,911 reads and 1,553,537 reads respectively (mean = 14,762, range = 3 to 43,942 reads/library for cloacal and mean = 27,255, range = 1 to 77,270 reads/library for esophageal). When detected, seabird reads ranged from four to 19,292 reads per library (mean = 388). The Greater Sage-Grouse "control" library in each sequencing run generated 470 reads in the cloacal sample sequencing effort and 49,264 reads in the esophageal sequencing effort. Under our "Max Contamination" detection threshold, any library with at least one read from a seabird was considered a detection. Our control Greater Sage-Grouse library contained zero seabird reads and no libraries beside the control library contained any Greater Sage-Grouse reads, suggesting low to nonexistent cross-library contamination.

We detected DNA from six seabird taxa in 12 of 112 swabs taken from 11 of 55 total sampled Barn Owls (range 0 to 4 taxa per swab, with four owls containing DNA from more than one seabird species; Table 2 and Supplementary Table 3). Of these 12 swabs, 7 were cloacal and 5 esophageal. There was just one case in which seabird DNA was found in both the cloacal and esophageal swabs taken from an owl (Sample 888; Supplementary Table 2). In this case, we detected the same species in the esophageal swab as the cloacal swab, but the cloacal swab detected two additional species. In all other cases, seabird DNA was detected in just one of the two swabs per individual.

Table 2. The number and source of seabird species detections in seven cloacal swabs and five esophageal swabs. Six swabs (cloacal swabs 1048 and 888, and esophageal swabs 1089, 1245, 858, and 888) contained seabird DNA that could not be taxonomically assigned to one species. Swabs negative for seabird DNA are not included here for brevity.

Prey item	Cloaca	Trachea
Anous minutus	1	0
Ardenna pacifica	4	1
Procellariidae spp.	2	4
Pterodroma sandwichensis	1	1
Puffinus newelli	2	1
Sula sula	1	1

Five avian prey DNA sequences could not be confidently assigned to species (cloacal swabs 1048 and 888, and esophageal swabs 1089, 1245, 858, and 888). These sequences are grouped under "*Procellariidae* sp.," as these sequences have the closest similarity to species within the family Procellariidae but are not similar enough to any species with a reference for a species-level assignment (percent identity with nearest family member represented in GenBank = 81.25 to 88.12). Three of these five sequences could belong to *B. bulwerii* as they differ by only one nucleotide respective to each other and were detected in an owl that had *B. bulwerii* morphologically identified in stomach contents. A reference sequence from this species is not available, thus we could not compare it to any of the unknown sequences. The *A. minutus* sequence had identical BLAST metrics as *Anous tenuirostris* but we identified it as *A. minutus* because *A. tenuirostris* is not present in the region (Raine et al. 2021). Other bird DNA recovered from swabs was that of the host, *T. alba* (all samples), wild turkey (*Meleagris gallopovo*; Individual 1171), and members of the Columbidae family (individuals 1088 and 891), all of which are known to occur within the study area.

Some samples had seabird sequences present in low abundance and it is possible that these are the result of sample cross-contamination. However, we believe that the patterns of detection and our contamination control library results suggest that these are real detections. These samples are swab specimens, so they inherently capture less DNA and therefore may have lower read counts than a tissue sample (Corthals et al. 2015, Handel et al 2006). For example, we detected *S. sula* in swab samples from one individual. Although there were very few of these target reads, they were present in both its cloacal swab (six reads) and esophageal swab (four reads), which underwent separate library preparation and sequencing. No other samples contained *S. sula* DNA, making sample-to-sample contamination highly unlikely. Furthermore, Barn Owls are known to depredate *S. sula* chicks on the Hawaiian Islands (Raine et al. 2019), providing more evidence that these detections are not from contamination, either field- or lab related, but truly derive from predator consumption.

Seabird detection in cloacal versus esophageal swabs

Cloacal swabs detected a greater number of seabird taxa (cloacal = 6, esophageal = 5); however, esophageal swab libraries produced more reads from seabird species. After filtering, there were 1,553,537 esophageal swab reads (excluding the positive control) of which 1,518,089 (97.7%) belong to *T. alba* and 31,901 (2.05%) belong to seabirds. For the cloacal swab libraries, 811,911 reads remained after filtering excluding the positive control. Of those reads, 799,751 (98.5%) belonged to *T. alba*, and 11,552 reads (1.42%) belonged to seabirds. Importantly, both types of swabs picked up seabird DNA when their respective pair did not. For example, *P. sandwichensis* DNA was detected in the cloacal swab for individual 973, but not in its esophageal swab.

Comparison of morphological and molecular diet assessment

There was only one case in which morphological assessment of stomach contents during necropsy led to a species-level identification of an avian prey item (individual 888; *B. bulwerii* skull; Supplementary Table 2). One owl had an unidentified bird in its stomach contents, but it could not be identified any further due to degradation; however, using molecular analysis of swabs, we were able to determine that this was *P. sandwichensis*. Six Barn Owl stomachs contained feathers, but these could not produce a species-level assignment or be conclusively identified as belonging to seabirds. The stomach contents for the other 47 owls were either empty, too degraded to identify, or consisted entirely of non-bird items, for example, insects, rats, etc. Thus, morphological assessment of owl stomach contents detected seabird species as prey items in 2% (1/55) of sampled owls whereas molecular analysis detected seabird species in 20% (11/55) of sampled owls (Fig. 2).

Discussion

Using molecular analyses of swab samples, native seabird species, including the endangered *P. sandwichensis* and threatened *P. auricularis newelli*, were detected in 20% of sampled Barn Owl diets. Previous diet studies of invasive Barn Owls in Hawai'i have been reliant upon the visual assessment and identification of prey items, either through analysis of stomach contents, pellet dissection, or observation of predation (Byrd and Telfer 1980; Work and Hale 1996; Mostello and Conant 2018; Raine et al. 2020). Based on our comparison with necropsy data, morphological approaches likely substantially underestimate the incidence of seabird predation: Metabarcoding of swab samples detected seabirds as diet items in 10 times more individual Barn Owls than with visual assessment of stomach contents. When considering the difficulty and rarity of finding Barn Owl-predated seabird carcasses and likely lack of large seabird remains in pellets, metabarcoding of Barn Owl swab samples can more easily provide insight into species-specific predation impacts which are otherwise difficult to observe.

Furthermore, it is important to contextualize these results within the larger framework of parameters that affect growth rate in long-lived populations. Adult survival has been shown to be the most sensitive parameter that influences growth rate in long-lived seabird populations, followed closely by pre-breeding survival (Doherty, Jr. et al. 2004). A similar trend was found in the long-lived seabird species, Heermann's Gull (*Larus heermanni*; Velarde and Ezcurra 2018). Barn Owls on Kaua'i and its neighboring islets have been documented primarily targeting adults for seabird prey, with one study revealing adults represented 94% of the 353 recorded seabird depredations where age was known or estimated, while chicks were only 6% of depredations (Raine et al. 2019). Certain seabird species, including *P. sandwichensis* and *P. newelli*, may even be predated by Barn Owls exclusively as adults (Raine et al. 2019, 2020). Although the results of our study do not provide age-class characteristics, it is possible that the Barn Owls described here exhibit similar patterns in seabird prey choice. By predating mostly adult seabirds, Barn Owls can influence the most sensitive parameter affecting population growth rate in these long-lived populations. This impact may be exacerbated in populations that are small; thus, the loss of just a few individuals may have a significant impact

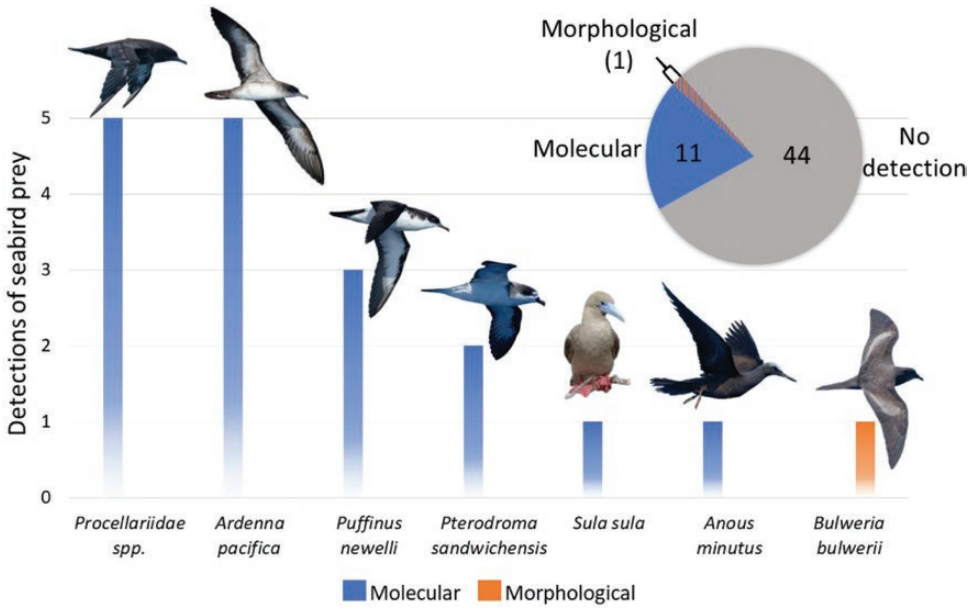

Fig. 2. Seabird prey detected in the diet of Barn Owls (n = 55) using molecular analysis of swab samples and morphological analysis of stomach contents. Bars represent prevalence of seabird prey in Barn Owl diets. Molecular analysis detected six seabird taxa as diet items in 11 owls, 4 of which had multiple seabird taxa detected. Species-level assignment of diet items was possible for 5 seabird species; however, 5 avian prey DNA sequences could not be confidently assigned to species and are collectively labeled as "*Procellariidae* spp.," due to their closest similarity to species within the Procellariidae family. Morphological analysis, in contrast, detected *Bulweria bulwerii* as a diet item in one owl. This same owl also had seabird prey detected using molecular methods, including taxa within the "Procellariidae" group. Given the unavailability of a reference sequence for *B. bulwerii,* it is likely that the molecular "Procellariidae" detection in this individual represents *B. bulwerii* as well. Photos courtesy of Alex Wang and PEN_ASH Photography.

on the ability of these seabird colonies to persist. Therefore, the continued removal of Barn Owls in Hawai'i may result in alleviating predation pressure on endangered seabird species and improvements in their population growth (Raine et al. 2019).

These results also revealed unexpected insights into potential seabird spatial presence on Kaua'i. Some species detected in these Barn Owl diets are either undocumented or uncommon in the areas where the Barn Owl was captured from (Fig. 1). In particular, three Barn Owl samples tested positive for Procellariidae species (potentially including *B. bulwerii*) in areas where such potential species are not known to be common. An additional sample tested positive for *P. sandwichensis* in an area where they have not been documented breeding. As Barn Owls were attracted to the hunting area using territorial Barn Owl calls, we can assume that captured owls are either looking for mates or defending territory and were captured within their home range (Nichols and Fuller 1987). A conservative estimate of Barn Owl home range, based on literature from various sources, is around 5.45 km^2, varying based on factors including vegetation density, prey availability, and ecosystem type (Byrd 1982; Colvin 1984; Hegdal and Blaskiewicz 1984; Rosenburg 1986; Taylor 2003; Thomsen et al 2014; Massa et al. 2015; Séchaud et al. 2022; Cain et al. 2023). The presence of these species in the Barn Owl diet, in combination with our limited understanding of owl home range, can provide managers with estimates of where such seabird species were present at time of capture. More research on the hunting and home range sizes of Barn Owls specifically in Kaua'i would help improve the accuracy of these assessments.

Additionally, the correlation between the time of capture of these owls and the specific seabird species found in their diet holds significant implications. Barn Owls were collected from late January through early December, with collection efforts focused during the breeding season of at least one seabird species (July to October). All Barn Owls that had seabird detected in their diet were captured from late April through late November, and each capture date was within the breeding season for the respective seabird species detected. Managers may choose to pair removal efforts with molecular analyses to better understand when in their breeding phenology certain seabird species are most vulnerable to Barn Owl predation and temporally and spatially focus higher levels of hunting efforts as a result.

When applying this tool in the future, our results suggest that swabbing both the esophagus and cloaca are likely to improve detection. Results revealed multiple instances where either esophageal swabs or cloacal swabs detected seabird DNA, but rarely both (Supplementary Table 2). As both swab types individually proved capable of resulting in a positive seabird detection when the other did not, the collection of both swab types is critical for future sampling protocols. Although studies have shown that Barn Owls have short digestive tracts and digestion times relative to other raptor species, it is still unknown how long prey DNA is detectable in either swab type (Barton and Houston 1996). Digestion in Barn Owls has been documented lasting 8 to 12 h in captive individuals, with pellet formation beginning at 6 h after consumption of prey (Smith and Richmond 1972; Barton 1992). While we do not fully understand the exact duration of DNA detectability within either swab type, we can make a few assumptions regarding temporal overlap between types. As many positive swabs—either cloacal or esophageal—did not have a positive complimentary swab sample, it is possible that there is minimal temporal overlap between the detectability of DNA between both swab types. However, it is also possible that the probability of detecting prey DNA from swab samples is low and that stochastic detection results in underestimating the true temporal overlap of detectability (Miles et al. 2015; Harper et al. 2023). Nonetheless, we can assume there is some temporal overlap, as seen in sample 888 which contained partly digested Procellariidae in the stomach as well as both swab types (see sample 888). Additionally, time of prey detectability may vary for each swab type based on a few factors. For esophageal swabs, DNA is introduced into the esophagus and oral cavity at time of prey consumption and potentially reintroduced in the event of pellet egestion. Reintroduction of prey DNA via pellet egestion may not occur in cases where the Barn Owl only consumes muscle tissue of larger prey items. For cloacal swabs, DNA is introduced into the small intestine just prior to first defecation of the digestible portions of the prey item and remains for an unknown duration, but some older fecal matter may be present around the cloacal opening containing DNA from prior meals. More research is needed to determine the time to defecation after prey consumption, as well as the length of time that prey DNA from the cloaca and esophagus is detectable following consumption. Such information would aid in better understanding timing and location of prey capture.

The effectiveness of molecular approaches can be optimized to generate more useful data depending on the specific research question. As in many diet metabarcoding efforts, predator DNA sequences composed a large portion of the sequencing effort (97.7% to 98.5% of swab sequences after filtering). This is difficult to avoid when the target prey taxa are closely related to the predator (i.e. in this case, birds). Some researchers have implemented blocking primers to reduce predator amplification (e.g. Pertoldi et al. 2021), but this has also been shown to create potential biases in resulting prey composition and interpretation when sequence divergence is low (Piñol et al. 2015). Although much of the sequencing effort was largely dedicated to Barn Owl reads, as stated above, we were able to successfully amplify seabird DNA without the use of blocking primers through the modifications of the MiBird primer set.

In a previous study of Barred Owl (*Strix varia*) diets in western North America the authors used a suite of 15 highly taxonomically focused primers to target specific potential prey taxa (Kryshak et al. 2022). This approach appears to have been effective at isolating specific diet items from within bulk gut contents, which was not possible using the more general primers in our study. However, this approach also necessitates a large amount of effort in terms of PCR enrichment and library preparation, which was one of the most expensive and time-consuming steps in the workflow for this study. Furthermore, since our focus was to determine the seabird species within Barn Owl diets, we chose to prioritize the detection of seabird prey at the cost of potentially missing other non-avian prey. Thus, our method generated data sufficient for the scope of this study in a short amount of time, as it required no prior in vitro testing of blocking primers and significantly less benchwork time than a multi-locus approach.

Another potential limitation of targeted enrichment approaches (e.g. multiple taxon-specific primer sets) is that these analyses constrain the scope of inference only to

"expected" taxa, missing some opportunities for discovery of previously undetected trophic interactions (Sarkis et al. 2022). The intermediate approach taken in this study sought to use a polymorphic locus with primers designed to bias amplification away from the predator species while being broadly applicable to other potential avian prey items. We note, however, that these approaches are not mutually exclusive. In some cases, a hybrid approach such as multifaceted (Swift et al. 2018) or multi-locus microfluidic (Hauck et al. 2019) metabarcoding that incorporates both taxon-specific and more conserved primers could be effective at both sensitively detecting rare priority taxa and detecting previously unknown diet items. A multi-locus approach could also potentially provide greater taxonomic resolution. Some of the seabird sequences in our study were assigned to "Procellariidae" only in part perhaps due to reference database incompleteness (*12S* sequences for *B. bulwerii*, *Puffinus bryani*, and *Pterodroma hypoleuca* were not available during this study) and also due to limited taxonomic informativeness of the *12S* locus; a less conserved locus may distinguish between *A. minutus* and *A. tenuirostris*, which were ambiguous in our dataset.

Although these methods appear to be highly accurate for detection of predation, a potential limitation of these molecular tools is that a single sample represents a relatively short snapshot in time. Furthermore, metabarcoding, particularly from swabs, is nonquantitative. We know that the owl ate a given prey species within a finite time period, but we do not know how many individuals of that species it consumed in the given time period, the regularity with which that species is consumed, what proportion of the diet is composed of this species, or the age class of the prey items it ate. Although in some systems metabarcoding read counts can provide inference about diet item proportions, the feeding ecology of owls and the avian prey in this study does not lend itself to these analyses. Barn Owls frequently consume larger prey, such as seabirds, as their only diet item per meal (Yom-Tov and Wool 1997). Thus, approximating proportions of diet items from digestive tract samples will likely not provide a good representation of the overall diet for a given animal. Furthermore, relative read abundance analyses typically require application of shared, conserved primers across diet items. To detect low-abundance avian prey items from the avian predator we used avian-focused primers and did not generate read counts from any non-avian diet items. Finally, we found that swab samples were more effective at detecting avian prey items than gut contents, but still, read counts for diet items were low. To our knowledge, no previous studies have attempted inference of relative species abundance from swab samples. Methods such as stable isotope analysis provide coarser taxonomic and temporal resolution but may also provide complementary information about the relative importance of seabirds to Barn Owl diets and integrate information over longer periods of time (Anderson and Polis 1998; Stapp 2002; Harper 2007; Pascoe et al. 2022). Identifying trends, or lack thereof, in the prey age classes that Barn Owls target would also help reveal the full impact they have on each seabird species' breeding potential and impacts on population growth.

Based on our findings we can confirm that Barn Owls on Kaua'i consume seabirds, both Threatened and Endangered, as well as more common species. Molecular metabarcoding of esophageal and cloacal swab samples is a technique that can be applied to identify seabird species in the diets of Barn Owls in otherwise difficult-to-access areas and seabird colonies. The method developed here has proven more effective than visual examination of the Barn Owl digestive tract, and likely reveals prey species in the diet that would otherwise be underestimated in traditional pellet-based diet studies. These results may aid managers in strategically planning Barn Owl hunting efforts to best protect these susceptible species.

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Supplementary Material

Supplementary material is available at *Journal of Heredity* Journal online.

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Conflict of Interest

AED is the Science Director and one of the co-owners of Hallux Ecosystem Restoration LLC which currently receives funding on contracts for predator control services for seabird conservation projects on Kaua'i. On these projects Barn Owl control is a service required by the contracts, as Barn Owls are documented killing threatened and endangered seabirds in Hawai'i.

Data Availability

The mitochondrial 12s sequences for *Pterodroma sandwichensis*, *Puffinus auricularis newelli*, and *Ardenna pacifica* generated by Sanger sequencing have been uploaded to GenBank (accession numbers: OR388121, OR388122, and OR388123). Sequence data resulting from 12s metabarcoding have been uploaded to the GenBank Sequence Read Archive under BioProject PRJNA1002395.

Author Contributions

YR, AED, MKS, and TMW conceived and designed the original study. AED coordinated all field sampling. JWE and TMW designed and conducted the molecular and bioinformatic analyses. JWE and TMW led writing of the manuscript, but all coauthors contributed to writing.

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Appendix A

Digestive tract sample preparation

Whole digestive tracts from *T. alba* were sent on dry ice to our facility (n = 31 animals). Upon arrival, samples were immediately stored at –20 °C until DNA extraction. Specimens were allowed to thaw at room temperature for 15 min prior to dissection and sample collection. Sterile scalpels were used to isolate the digestive tract from other organs if present and to create incisions along the stomach and at the bottom of the intestines. Solid contents of the stomach were lightly scraped from the surface to reduce collection of predator DNA, and liquid from the intestines was squeezed out and collected in a 50 mL tube. DNA was then extracted from this combined material using Qiagen DNeasy Blood and Tissue kit following the manufacturer's protocol (Qiagen Inc., Valencia, California). All downstream processes are identical to those applied to swab samples, except for an additional clean-up step prior to PCR using Zymo OneStep PCR Inhibitor Removal kit (Zymo Research Co., Irvine, California).