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MetaBARFcoding: DNA-barcoding of regurgitated prey yields insights into Christmas shearwater (*Puffinus nativitatis*) foraging ecology at Hōlanikū (Kure Atoll), Hawai'i

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Abstract

Morphological identification of digested prey remains from a generalist predator can be challenging, especially when attempting to match degraded remains to taxonomic keys. DNA techniques, whereby prey is sequenced and matched to large public nucleotide sequence databases, are increasingly being used to augment morphological identification. We used "metaBARFcoding" (DNA metabarcoding) to target a region of the cytochrome *c* oxidase subunit I (COI) mitochondrial gene to identify prey in highly digested regurgitations from Christmas shearwaters *Puffinus nativitatis* at Hōlanikū (Kure Atoll). Metabarcoding was used to bulk-process 92 water samples from regurgitations collected from 2009 to 2017, providing an overview of the seabird's diet. We additionally Sanger-sequenced 100 prey items from 50 randomly chosen regurgitations to verify that metabarcoding characterized key components of the diet. The metabarcoding technique identified 87 unique taxa from 29 families of fish and squid, spanning diverse taxa, including reef-associated, pelagic–oceanic, and mesopelagic species. Rare prey (frequency of occurrence ≤5% of samples) constituted 66% of the species richness, demonstrating the highly diverse diet of this generalist predator. Overall, 81% of the families detected in the contemporary diet were previously documented in Christmas shearwater diets from the Northwestern Hawaiian Islands. Our results indicate that metabarcoding the COI region is useful in identifying a wide range of taxa from highly digested regurgitations, thus facilitating this approach to study seabird diets.

KEYWORDS
cytochrome *c* oxidase subunit I (COI), diet, DNA metabarcoding, eDNA, food web, mitochondrial DNA, seabird

1 | INTRODUCTION

The seas surrounding tropical seabird colonies are oligotrophic and characterized by patchy prey distributions (Ainley & Boekelheide, 1983; Ballance & Pitman, 1999). Some seabirds, notably those in the order *Procellariiformes*, are adapted for the challenge of searching vast areas for highly dispersed food (Ballance et al., 1997; Spear & Ainley, 1997; Spear et al., 2007). Morphologically, petrels and shearwaters that remain in the tropics year-round have large wings with high aspect ratios for energetically efficient gliding flight (Hertel & Ballance, 1999; Spear et al., 2007). Many tropical seabirds also depend on schools of sub-surface predators that drive prey to the surface and into the air, increasing accessibility to aerial predators (Ashmole & Ashmole, 1967; Ballance et al., 1997; Hebshi et al., 2008). As a result, the diets of tropical seabirds are quite diverse, because these opportunistic predators feed on a variety of shared prey, partitioned by size and vertical position in the water column (Donahue et al., 2021; Harrison et al., 1983; Spear et al., 2007).

Tropical seabirds remain at sea for extended periods of time during the breeding season, with foraging trips to provision their chicks lasting multiple days (Amerson and Shelton, 1976; Ashmole & Ashmole, 1967; Baduini & Hyrenbach, 2003). Ingested food breaks down while birds are at sea, producing high-energy stomach oil and degrading the morphological characteristics of the prey items (Ashmole & Ashmole, 1967; Carreon-Martinez et al., 2011; Moran et al., 2015). When researchers collect regurgitations at the colony, prey may be so digested that it is unrecognizable (Ashmole & Ashmole, 1967; Carreon-Martinez et al., 2011; Scribner & Bowman, 1998). Thus, the inability to identify digested prey to species level can lead to the loss of diet information critical to understanding marine food webs (Aguilar et al., 2017; Symondson, 2002). Moreover, generalist foragers that consume taxonomically similar groups can further hinder species-level identification (Symondson, 2002). DNA barcoding and metabarcoding techniques, whereby prey are sequenced and matched to large public nucleotide sequence databases for species identification, are increasingly being used to augment traditional morphological identification (Alonso et al., 2014; Deagle et al., 2007; de Sousa et al., 2019; Symondson, 2002).

Highly degraded prey from marine predators has been successfully identified using a variety of genetic methods (de Sousa et al., 2019; Symondson, 2002; Traugott et al., 2021). The DNA barcoding approach involves processing tissues collected individually from prey items and amplifying targeted DNA fragments with polymerase chain reaction (PCR), followed by Sanger sequencing (Hebert et al., 2003). This approach has been demonstrated successfully in degraded diets from seabirds (Alonso et al., 2014), sharks (Barnett et al., 2010), piscivorous fishes (Aguilar et al., 2017; Oyafuso et al., 2016), and cetaceans (Best et al., 2014). However, sampling tissues from individuals within highly degraded prey samples can distort the apparent diet, by over-representing less digested (e.g., larger or more recently ingested) specimens and under-representing small prey items and rare species (Carreon-Martinez et al., 2011; Deagle et al., 2009).

Next-generation high-throughput (HTS) sequencing approaches are being used to address this potential selection bias in diet studies, by using PCR to amplify and sequence standardized "DNA barcode" regions from a mixed sample at high sequence read counts to capture even the rarest of organisms present in the sample (Pompanon et al., 2012; Porter & Hajibabaei, 2018; Taberlet et al., 2012). Not only does HTS provide a more comprehensive assessment of prey taxonomic richness, but it can also be less costly and time-consuming than morphological diet analysis (Pompanon et al., 2012). High throughput, environmental DNA (eDNA) metabarcoding approaches are increasingly common for processing water and sediment to assess marine biodiversity (reviewed by Eble et al., 2020). Similar approaches can be taken to bulk-process degraded diets (dDNA) of organisms using gut contents, regurgitations, or feces (Ando et al., 2020; Corse et al., 2010; Deagle et al., 2005, 2009; de Sousa et al., 2019; Nalley et al., 2021; Smith et al., 2005). HTS methods may be complemented by Sanger sequencing to augment information from bulk-processed digested DNA, and specifically provide identification of individual prey items in diet samples.

The goal of this study was to identify the contemporary diet of an understudied tropical pelagic seabird, the Christmas shearwater (in Hawaiian 'Aoʻū) *Puffinus nativitatis* (CHSH), using "metaBARFcoding," the opportunistic collection and processing of regurgitations (barfs) using a dDNA approach (de Sousa et al., 2019). Random individual prey items from 50 of the regurgitation samples were also Sanger-sequenced to verify that the metabarcoding technique identified key prey species within the diet. Finally, the species richness derived from metabarcoding was compared to the results from a historical morphological identification study of CHSH diet in Hawaiian waters (Harrison et al., 1983).

2 | MATERIALS AND METHODS

2.1 | Sample collection

We obtained diet samples from a CHSH colony on Hōlanikū (Kure Atoll, 28°25′N, 178°20′W), the Hawaiian Island furthest to the northwest, and located within the Papahānaumokuākea Marine National Monument (PMNM). CHSH breed on an annual cycle, where from April to early May, CHSH are at the colony and laying eggs, which hatch in May through June. Chicks are fed by both parents until they fledge in September through October, at which point adult birds remain at sea until the breeding cycle begins again. While the birds were attending the colony, the breeding status of individuals was not certain, but we assumed these birds were breeding based on the stage of the annual breeding cycle.

Samples were opportunistically collected between April and September of 2009, 2011, 2013, 2015, and 2017 from adult birds during an annual population mark-recapture study (VanderWerf et al., 2015). For mark-recapture, birds were netted on the ground (coral rubble), and often voluntarily regurgitated onto the ground prior to handling, providing an opportunistic barf sample for diet

analyses, but making sterile collection difficult. Immediately after a bird regurgitated, samples were collected using gloves and placed into individual labeled plastic bags that were filled with fresh reverse-osmosis (RO) drinking water. Samples were frozen until analysis in the lab on O'ahu, Hawai'i.

2.2 | Sample sorting

Samples were thawed, then whole prey items and parts were pulled out of the RO water mixture for sorting. The remainder of the sample was passed through a 500 μm sieve. The material retained in the sieve was sorted into categories: "fish," "squid," and "mush" (indistinguishable tissue sized >500 μm), following Donahue et al. (2021). Everything that passed through the sieve, including the 200 ml of RO water in which the regurgitation was frozen, was transferred to a labeled sterilized container and used as the starting material for DNA metabarcoding analysis.

We used a weighted average freshness (WAF) metric to quantify the degree of digestion of the diet based on the freshness of each prey item per sample on a scale of 1 (fresh and intact prey item) to 3 (highly digested prey item or parts; Donahue et al., 2021). We calculated the WAF of each regurgitation by averaging the freshness value of each individual prey item or their parts, weighted by masses following the formula:

$$\mathrm{WAF} = \left(\sum \left(M_{\mathrm{Pi}} * F_{\mathrm{Pi}}\right) + \left(M_{\mathrm{Fp}} * F_{\mathrm{Fp}}\right) + \left(M_{\mathrm{Fp}} * F_{\mathrm{Fp}}\right) + \left(M_m * F_m\right) / (\mathrm{TM}) \right)$$

where M represents mass and F represents the freshness rank for the different prey constituents: Pi: prey item, Fp: fish parts, Sp: squid parts, m: mush. TM is the total mass of the sample: $\sum \left(M_{\mathrm{Pi}}\right) + M_{\mathrm{Fp}} + M_{\mathrm{Sp}} + M_m$. The unitless WAF index ranges between 1 and 3, with 1 representing a fresh sample and 3 representing a highly digested sample.

2.3 | DNA metaBARFcoding

2.3.1 | DNA extraction

The regurgitations were originally collected without any intention to conduct molecular analyses, hence the collection and freezing in RO water, which is standard protocol for seabird diet studies (e.g., Nimz et al., 2021). The intact prey items were required for another study, but we processed the remaining tissue (<500 μm in size) and the samples' RO storage water as the basis for our molecular diet investigation. The RO water from each regurgitation sample (200 ml) was aliquoted into a 50-ml Falcon tube and pelleted by centrifugation at 2510 g for 10 min at room temperature. The supernatant was decanted without disturbing the pellet, and additional sample liquid was added to the tube, repeating the centrifugation and decanting process until the entire regurgitation sample (200 ml) was pelleted in a single 50-ml Falcon tube. Following the final centrifugation and removal of liquid, the tubes were inverted for at least 1 h to air dry.

To digest the pelleted material, 3 ml of Longmire's buffer (Longmire et al., 1997) and 120 μl of Proteinase-K (20 mg/ml) were added to each tube; samples were vortexed for 3–5 s and incubated overnight at 37°C.

Following incubation, DNA was extracted from a 700 μl volume of each digested regurgitation via phenol-chloroform-isoamyl alcohol (PCI, 25:24:1) and chloroform-isoamyl alcohol (CI, 24:1) phase-separations (Renshaw et al., 2015). Sterile water (100 μl) was added to each tube to rehydrate the pelleted DNA extractions. Potential inhibitors in the DNA extractions were removed using Mag-Bind® TotalPure NGS (Omega Bio-Tek Inc) magnetic beads at a volume ratio of 1.2 (beads):1 (DNA extract) following the manufacturer's recommendations. Additional details for DNA extractions are in the Appendix S1.

2.3.2 | Selection of primers

Prior to this work, almost all seabird diet studies using a metabarcoding approach used feces as the template, rather than stomach contents or regurgitations. Doyle and Adams (2018) attempted metabarcoding of Buller's Shearwater (*Puffinis bulleri*) regurgitations using a portion of the 16s ribosomal gene region with minimal success. Therefore, a random subset of samples ($n = 32$) were used in an initial experiment to identify the optimal primer sets to use for the full analysis since using all assays on the full dataset was cost-prohibitive. Six published primer assays were tested: Ac16S-F/-R, Am12S-F/-R, F-574/R-952, jgHCO1490/mlCOIintR, mlCOIintF/jgHCO2198, and BF1/BR2 (details in Table 2). Assays to test were chosen based on prey taxa listed in previously published studies on the diet of CHSH (Harrison et al., 1983), and on prey taxa identified in the initial morphological sorting of diet items. All assays had publicly available DNA sequence data for the primary prey groups, fish, and squid.

From the initial primer assay test, we selected two primer assays to use for all samples ($n = 95$): mlCOIintF/jgHCO2198, and BF1/BR2 (Table 2). The Illumina library preparation steps are described below for the two primer assays used on all samples, but all library preparation steps were the same for both the six primer assay test and the full analysis with two primer assays.

2.3.3 | Illumina library preparation and sequencing

Two rounds of polymerase chain reaction (PCR) were used to amplify targeted fragments from DNA extracts and prepare libraries for Illumina sequencing following Olds et al. (2016). Briefly, for the first PCR, each DNA extract was PCR amplified at each assay (mlCOIintF/jgHCO2198, BF1/BR2) with a portion of the Illumina adaptor sequence included on the 5' end of the synthesized primer pairs. The 20 μl PCR mixes consisted of: 4 μl of 5× GoTaq Flexi Buffer (Promega), 0.4 μl of 10 mM dNTPs, 1.6 μl of 25 mM $MgCl_2$, 1 μl of 10 μM forward primer, 1 μl of 10 μM reverse primer, 8 μg of Bovine

Serum Albumin (BSA, 20 mg/ml), 0.15 μl of GoTaq G2 Flexi DNA Polymerase (Promega), 4 μl of DNA extract, and 5.85 μl of sterile water. PCR cycling protocols followed those used for each primer set in the respective manuscripts cited in Table 2. PCR products from the first round of PCR were cleaned with Mag-Bind® TotalPure NGS (Omega Bio-Tek Inc) magnetic beads at a ratio of 1 (beads):1 (DNA), following the manufacturer's recommendations. DNA concentrations for PCR products were then quantified with the Qubit dsDNA HS Assay (Life Technologies).

The second round of PCR added library-specific dual-indexes using Nextera XT v2 adapters following Illumina's protocol, with the PCR products from the first round of PCR as the template DNA (see Appendix S1 for details). PCR products with the adapters added were cleaned with Mag-Bind® TotalPure NGS (Omega Bio-Tek Inc) magnetic beads at a ratio of 0.8 (beads):1 (DNA) following the manufacturer's recommendations. Individual library DNA concentrations were quantified with the Qubit dsDNA HS Assay (Life Technologies. Libraries were 300 bp paired-end sequenced on an Illumina MiSeq at the University of Notre Dame's Genomics and Bioinformatics Core Facility (http://genomics.nd.edu) with a MiSeq Reagent Kit v3 (600-cycle, Illumina). The Core Facility pooled equimolar concentrations of all libraries and added a 25% Phi-X DNA spike-in for the sequencing run. One full MiSeq flow cell was used to test the six primer assays on 32 regurgitation samples. The metabarcoding of the full set of 95 regurgitation samples at the two selected primer assays was spread across three MiSeq flow cells, but at a total target level of one additional MiSeq flow cell's worth of data. Additional details for Illumina library preparation and sequencing can be found in the Appendix S1.

2.3.4 | Mock community

We incorporated a mock community sample in our study design to serve as a positive PCR control and a quantifiable measure of sample cross-contamination, as described in Deiner et al. (2017). For our mock community, a 500-ml water sample was collected from aquaria housing only two species of clownfish, the tomato clownfish *Amphiprion frentatus* and the Ocellaris clownfish *Amphiprion ocellaris*, in the finfish department at Oceanic Institute of Hawai'i Pacific University. The mock community water sample was vacuum filtered through a single 47 mm GN-6 Metricel 0.45 μm MCE membrane disk filter (Pall Corporation, New York). The filter was submerged in 700 μl of Longmire's buffer (Longmire et al., 1997) in a 2 ml microcentrifuge tube and stored at room temperature. DNA extraction for the mock community sample followed the protocol outlined in Renshaw et al. (2015), starting with the phenol–chloroform–isoamyl alcohol (25:24:1) step. The Illumina library prep for the mock community sample was performed alongside the regurgitation samples to serve as a positive PCR control and to monitor for contamination or miss-tagging (i.e., clownfish sequences in the regurgitation libraries or vice versa), since clownfish species do not occur in the Hawaiian Islands.

2.3.5 | Bioinformatics pipeline

Species identifications in regurgitation samples followed the clustering of MiSeq read output into Operational Taxonomic Units (OTUs). The quality filtering of reads and clustering into OTUs followed steps outlined in Olds et al. (2016) and Li et al. (2018). A reads table (number of reads per OTU per sample) and a fasta file of centroid sequences from each OTU were output. Additional details for the generation of OTUs can be found in the Appendix S1.

Using the BLAST + toolkit (Camacho et al., 2009), megablast was run locally to match OTU sequences to records in the nt_v5 database (downloaded July 29, 2020). TaxId output from BLAST searches was matched to lineages (kingdom, phylum, class, order, family, genus, and species) using the custom script *taxid2lineage.py* (available at https://github.com/setoews/NCBI_ftp/tree/master/taxID%20conversions).

To account for potential contamination (field or lab-based) or miss-tagging, samples were filtered using the mock community read counts as non-arbitrary thresholds (i.e., Corse et al., 2017). The OTU-specific incidence rate (number of reads in a single library/total number of reads) for OTU detections shared between the mock community sample library and each of the regurgitation libraries was calculated for each OTU for each primer set. Read numbers below a primer-specific threshold (the highest observed incidence rate) were set to zero. Subsequent analyses focused only on remaining OTUs that matched to fish (Metazoa, Chordata, Actinopteri) or squid (Metazoa, Mollusca, Cephalopoda) taxa. Final taxonomic assignments for OTUs were based on Query % and Identity % BLAST matches. A ≥99% Query match was required for all assays. The Identity % match varied by assay, with the 12S, 16S, and 18S assays all requiring a ≥99% match for the primer test run, and the cytochrome oxidase *c* subunit I (COI) assays requiring a ≥97% match for species identification in the primer test run and (for Leray2 and B12) the full analysis. A less-stringent match-criteria was also considered for COI sequences (≥90% Identity) to attempt to identify OTUs at the family level in cases with no species-level match.

2.3.6 | Metabarcoding verification

To verify that metabarcoding identified common species within the CHSH diet, we also sequenced 100 randomly selected individual prey items from regurgitations of 50 CHSH, following the Sanger sequencing methods described in Nimz et al. (2021).

2.4 | Data analysis

2.4.1 | Comparing prey mass with prey diversity (via metabarcoding)

Because we were using opportunistically collected regurgitations that varied widely in mass and the number of prey items, we explored

potential biases in the number of species detected, as a function of the mass of the individual samples. We performed contingency tests (X^2) to assess differences between prey categories using R 3.5.2 (R Core Team, 2020), with significance assessed at $\alpha = 0.05$.

2.4.2 | Prey species richness

Species richness was assessed using sample-based rarefaction curves (Gotelli and Colwell, 2011). We used the Chao 1 estimator (correcting for the presence of rare species, singletons, and doubletons), and the Chao 2 estimators (correcting for the incidence of rare species) using EstimateS biodiversity software (version 9.1.0, Colwell, 2013).

3 | RESULTS

3.1 | Prey type, occurrence, and mass

In total, 95 regurgitation samples comprising a total of 892 individual prey items and 1706 g (wet mass) were sorted and enumerated within the three categories of "fish," "squid" and "mush." Fish and squid accounted for 77% and 23% of the prey items, respectively (Table 1). Fish was the most common prey type, with a frequency of occurrence (FO) = 87% (SD = 3.5) of the samples. Squid and mush each had an FO = 74% (SD = 4.5) of the samples. The overall FO of fish and squid in the samples was significantly different ($X^2 = 5.672$, df = 1, $p = 0.017$).

None of the samples contained "fresh" (level 1) prey items. The vast majority of fish (99%, $n = 688$) and squid (96%, $n = 205$) were "severely digested" (level 3). Subsequently, the mean WAF (scale from 1 to 3) for all samples was 2.9 (±0.2 SD, $n = 95$, range = 2–3), reflecting severe digestion.

The mean number of prey items per sample was 9.4 (±7.3 SD, median = 8, range = 0–34, $n = 95$), with a larger average for the fish (7.2 ± 7.8 SD, median = 5, range = 0–34, $n = 205$) than for the squid (2.2 ± 2.3 SD, median = 2, range = 0–9, $n = 95$). Overall, fish and squid (including individual prey items and parts) accounted for 47% (801.7 g) and 37% (628.1 g) of the wet mass, respectively. The unidentifiable "mush" accounted for the remaining 16% (276.2 g) of the wet mass. Fish and squid did not occur together in all samples, as 23 samples only contained fish and 13 samples only contained squid.

3.2 | Genetic prey identification

3.2.1 | Primer test results

For our initial experiment to determine which primer sets to use for all samples, DNA was amplified in a subset of 32 randomly selected regurgitation samples using six primer sets targeting four gene regions: 16S, 12S, 18S, and COI (Table 2). After demultiplexing, a

TABLE 1 Summary of opportunistic regurgitation samples collected from Christmas shearwater adults (*Puffinus nativitatis*) on Hōlanikū from 2009 to 2017 ($n = 95$)

Year	Month	Breeding stage	Total No. of samples	Total No. prey items	Mean (SD) No. Prey items per sample	No. Squid prey items	No. Fish prey items	% Mass squid	% Mass fish	% Mass mush	Total mass (g)
2009	June	Early chick	26	409	15.7 (8.9)	13	396	6.3	53.9	39.8	535.7
2011	August	Mid chick	3	21	7.0 (4.0)	4	17	55.0	45.0	0	61.4
2013	April	Early egg	4	9	2.3 (1.7)	9	0	99.3	0.1	0.6	56.8
2013	June	Early chick	7	38	5.4 (4.0)	19	19	83.7	13.8	2.5	96.6
2015	June	Early chick	7	43	6.2 (2.3)	40	3	97.4	0.4	2.1	89.0
2015	Sept.	Fledging	28	198	7.1 (3.6)	91	107	46.6	48.9	4.5	497.7
2017	June	Early chick	11	69	6.3 (4.7)	13	56	36.0	51.4	12.8	146.2
2017	July	Early-Mid chick	6	74	12.3 (9.6)	16	59	31.6	59.8	8.5	158.9
2017	August	Mid chick	2	22	11.0 (1.4)	0	22	0.1	99.8	0.1	52.2

Note: Breeding stage represents the different developmental stages of the hatchlings when CHSH are at the colony from the early chick stage right after the hatchlings emerge in May through June to the time the hatchlings fledge in September through October. In some seabirds, foraging strategy changes and prey diet shifts during this transition.

TABLE 2 Summary of the primer sets used in the initial primer selection experiment (n = 32 diet samples) to determine which primer sets to use for metabarcoding of prey in Christmas shearwater (*Puffinus nativitatis*) regurgitations

Primer Set	Citation	Gene region	Name	Amplicon size (bp)	# paired-end reads	# OTUs (BLAST)	# OTUs (metazoan)	# OTUs (squid)	# OTUs (fish)
Ac16S-F/-R	Evans et al., 2016	16S	Ac16S	334–337	595,549	35	35	0	34 (19)
Am12S-F/-R	Evans et al., 2016	12S	Am12S	240–247	1,744,386	88	81	0	83 (39)
F–574/R–952	Hadziavdic et al., 2014	18S	Hadz	354–355	830,315	427	66	0	27 (8)
jgHCO1490/ mlCOIintR	Leray et al., 2013	COI	Leray1	319	1,496,753	2148	59	3 (3)	37 (24)
mlCOIintF/ jgHCO2198	Leray et al., 2013	COI	Leray2	313	3,917,827	2616	148	4 (4)	64 (44)
BF1/BR2	Elbrecht & Leese, 2017	COI	B12	316	3,612,085	1689	224	4 (4)	88 (58)

Note: The total number of OTUs are listed for the initial BLAST hits (BLAST), after filtering for only metazoan sequences (metazoan), after filtering for only squid (squid) and for only fish (fish). For OTUs matching squid and fish, the number of OTU taxa meeting the identity match thresholds of ≥97% (12S, 16S, and 18S) and ≥99% (COI) are also shown in parentheses. Amplicon size does not include primer regions. The shaded gray markers yielded the highest numbers of OTUs after match thresholds were applied, and detected both fish and squid taxa, so were used to analyze the entire sample set.

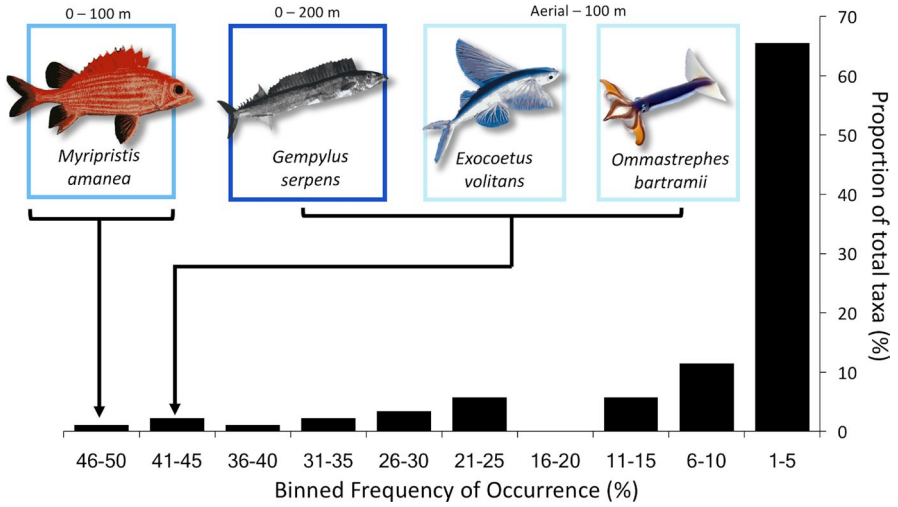

FIGURE 1 Binned frequency of occurrence (%) of prey (87 taxa, 68 species) identified to a greater than 98% match from Christmas shearwater (*Puffinus nativitatis*) stomach samples (n = 92). Images are of the four species that occurred in greater than 40% of the samples, with colored boxes distinguishing the oceanic depth range for the species as documented in FishBase (http://www.fishbase.org; Froese & Pauly 2021): aerial–100 m (light blue), surface–100 m (medium blue), surface–200 m (royal blue), and 200–1000 m (dark blue)

total of 12,196,915 paired-end reads were retained for all primers. Despite Qubit quantifications and equimolar pooling in the library preparation stages, demultiplexed paired-end read numbers varied across primer sets, from 595,549 reads for AC16s-F/AC16s-R to 3,917,827 reads for mlCOIintF/jgHCO2198 (hereafter referred to as Leray2) (Table 2; Table S1). Primer sets Leray2 and BF1/BR2 (hereafter referred to as B12; 3,612,085 paired-end reads), both targeting the COI region, identified the highest quantity of unique metazoan taxa (Table 2, highlighted gray) including both fish and squid. The primer sets targeting the COI region were the only assays that detected any squid taxa, and since the Leray2 and B12 both performed better than the jgHCO1490/mlCOIintR (Leray1) primer set that targeted COI, we chose the Leray2 and B12 primer sets to metabarcode all samples.

3.2.2 | Full analysis results

A total of 95 regurgitation samples were metabarcoded using B12 and Leray2 (Table 2). Three samples from the original 95 regurgitations did not amplify, and were discarded, and 92 samples were used in the analysis. After demultiplexing, 4,161,438 paired-end reads were retained for B12 and 6,002,147 paired-end reads were retained for Leray2. Summary statistics for each primer set can be found in Table S2. The B12 data resulted in a total of 5875 OTUs, with 4452 having a BLAST match and 445 identified as a metazoan. The Leray2 data resulted in a total of 8053 OTUs, with 7192 having a BLAST match and 388 identified as metazoan. Non-metazoan sequences, including bacteria, fungi, and plants, were discarded. The Leray2 primer assay provided an average of 99,375 (±120,202 SD,

median = 58,511, range = 8717–744,998) sequences per regurgitation sample, and B12 provided an average of 56,019 (±45,143 SD, median = 42,619, range = 0–187,417) sequences per sample. The two primer sets identified 62 taxa in common, with Leray2 identifying an additional 17 unique taxa and B12 identifying an additional 15 unique taxa. A contingency test identified significant differences between the two primer sets (Leray2 and B12) and the two prey categories (fish and squid) ($X^2 = 185{,}730$, df = 1, $p < 0.001$). Therefore, we combined the taxa identified by each of the two markers into a comprehensive list and used only the presence data for subsequent analysis rather than using the sequence abundances as a proxy for prey abundance.

The COI gene was successfully amplified for a wide range of taxa, but only classes Actinopteri and Cephalopoda were considered in our analysis of CHSH primary prey, following previous taxonomic analyses (Ashmole & Ashmole, 1967; Harrison et al., 1983; Spear et al., 2007) and our findings from sorting the prey into morphological categories. We were able to distinguish 68 species and 61 genera, within 29 families and 16 orders. The remaining 19 unique taxa were assigned to family level. On average, each sample contained 6.0 (±3.0 SD, median = 5, range = 1–13) distinct taxa. Including sequences identified only to family level, each sample had 8.7 (±3.0 SD, median = 8, range = 1–18) unique taxa. Overall, the most frequently detected species were the fishes *Myripristis amanea* (46%, Holocentridae), *Gempylus serpens* (41%, Gempylidae), and *Exocoetus volitans* (41%, Exocoetidae), and the squid *Ommastrephes bartramii* (42%, Ommastrephidae) (Figure 1). A comprehensive list of all detected species is included in the Table S3.

We performed quantitative analyses of the diet at family level rather than at the species level because 70% of the species were rare (FO ≤ 5% of the samples). When we calculated FO of prey families to identify important groups, Exocoetidae (represented by 10 species) tied with Ommastrephidae (represented by 4 species) as the two most common families in CHSH diet, both occurring in 78% of the samples (Figure 2). Holocentridae (represented by 10 species, including the most frequently occurring fish, *Myripristis amanea*) was the next most common family, occurring in 55% of the samples. While the family Mullidae (represented by 7 species) was also common (FO = 48%), these species did not have the same high occurrence when considered individually. Nearly half (48%) of the families were rare (FO ≤ 5% of the samples). Prey families live in a wide range of depths, from above the surface to below 200 m. We categorized general functional groups based on the oceanic depths in which the families are commonly found (www.fishbase.org; 2021): aerial–100 m, 0–100 m, 0–200 m, and 200–1000 m (Figures 1 and 2).

3.3 | Comparing prey mass with prey diversity

We explored if the mass of the squid and fish prey classes were correlated to the number of fish and squid species identified by metabarcoding. The mass of fish and squid were weakly, yet significantly, correlated with the number of fish and squid species detected by metabarcoding (fish: $r = +0.24$, df = 90, $p = 0.02$; squid: $r = +0.35$, df = 90, $p < 0.001$). The mass of the mush was not significantly correlated with the number of fish species detected by metabarcoding (all species: $r = -0.13$, df = 90, $p = 0.23$; fish: $r = -0.03$, df = 90, $p = 0.77$), but was negatively correlated with the number of squid species (squid: $r = -0.32$, df = 90, $p = 0.002$).

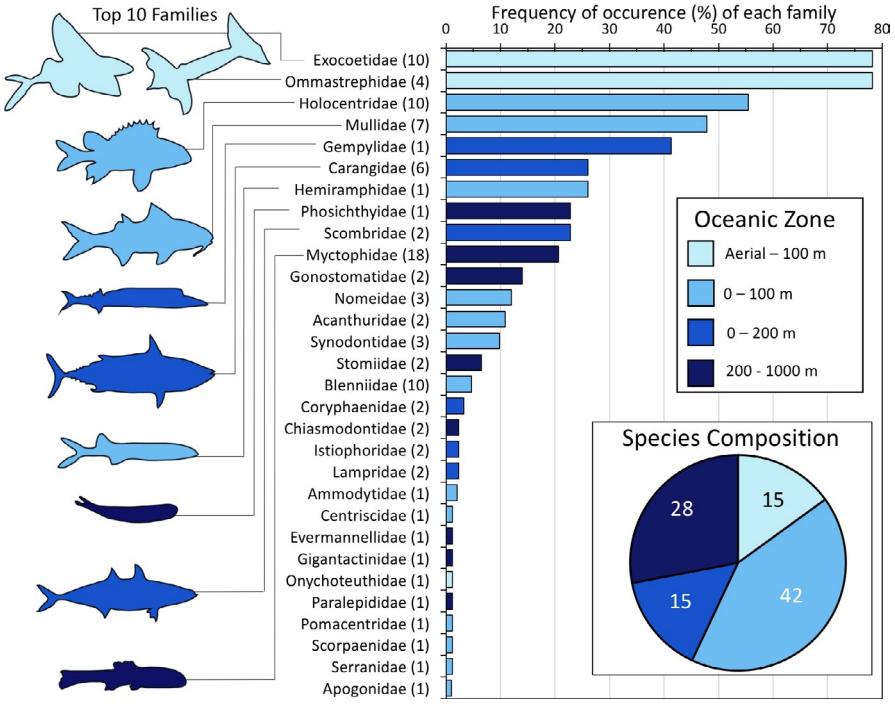

FIGURE 2 Frequency of occurrence (%) of prey family within diet samples ($n = 92$), derived from genetic analysis of the diet. Colors represent the four common oceanic depth ranges for the families according to FishBase (www.fishbase.org; Froese & Pauly 2021): aerial–100 m (light blue), surface–100 m (medium blue), surface–200 m (royal blue), and 200–1000 m (dark blue). The number of species identified within each family is shown in parentheses. Icons represent the top 10 families with >20% frequency of occurrence. The pie chart summarizes species composition by oceanic depth zone

3.4 | Mock community filtering

For the mock community sample, a total of 29,840 and 33,834 paired-end reads were generated from the B12 and Leray2 assays, respectively. Rates of incidence of 0.000357 and 0.000010 were estimated for the B12 and Leray2 assays, respectively, and applied as thresholds across each individual OTU, with read numbers below this threshold changed to zero on an individual sample basis.

3.5 | Prey species richness

Sample-based rarefaction analysis of 87 unique taxa indicated that the prey species richness was fully characterized by our samples (Figure 3). Both the S(est) and the Chao 1 & 2 estimators reached asymptotes. The expected number of species within the pooled samples (S(est)) fell within the 95% confidence intervals, but had a lower species threshold than the Chao 1 and Chao 2 richness estimators. The lower bounds of the Chao (1 & 2) 95% confidence intervals overlapped with the S(est) 95% confidence intervals, at approximately 70 samples and 80 species.

3.6 | Metabarcoding validation

Of the 100 prey items (within 50 samples) that were Sanger-sequenced, 76 prey items (from 30 samples) were also identified in their associated metabarcoded sample. The 24 prey items that were detected via Sanger sequencing of intact prey, but were not detected in the metabarcoding data for the same regurgitation sample, were prey species that were common in the overall diet, occurring in at least 5% of the regurgitations.

4 | DISCUSSION

4.1 | Contemporary diet

The combined metaBARFcoding, barcoding, and morphological data indicate CHSH is a generalist top-predator in marine pelagic systems, impacting fishes and squid species from the epipelagic and mesopelagic open ocean zones in addition to reef fishes in the juvenile stage of their biphasic life-cycles. Flyingfishes (Exocoetidae) and flying squids (Ommastrephidae) were the two dominant families in the CHSH diet, suggesting that despite being capable divers, CHSH primarily consumed these aerial prey at or above the surface. Thus, we hypothesize that CHSH associate with sub-surface predators that enable access to these epipelagic prey (Ballance & Pitman, 1999; Spear et al., 2007). Reef fishes (Holocentridae, Mullidae) were the second most common prey. Many of these fishes are nocturnal and reef-associated during their adult stage, but little is known about the vertical distributions of the pelagic juveniles. Larval reef-fish associate with surface convergence zones (Gove et al., 2019; Whitney et al., 2021), and juvenile forms are likely transitioning from these pelagic slicks to reef settlement. Juvenile reef fish have previously been documented in diets of fishes schooling with tuna in the open ocean (Choy et al,, 2013; Himmelsbach, 2021), and in the diet of tropical seabirds foraging over tuna schools, so they are likely captured in oceanic waters, rather than coastally (Nimz et al., 2021; Spear et al., 2007).

Seven fish families in the CHSH diet (Chiasmodontidae, Evermannellidae, Gonostomatidae, Myctophidae, Paralepididae, Phosichthyidae, and Stomiidae) are mesopelagic, generally occurring between depths of 200 and 5000 m during the day, and vertically migrating to the epipelagic zone at night (Hully, 2002). Similarly, snake mackerel (*Gempylus serpens*, family Gempylidae) are distributed from the surface to mesopelagic depths (600 m).

A concurrent diving study of breeding CHSH from this same colony recorded a maximum CHSH depth of 24 m (Nimz et al., 2021), indicating the daytime depths of these fish species are beyond the CHSH diving range most of the time. Nimz et al. (2021) did not document nighttime diving for any of the tagged CHSH breeding at Hōlanikū. However, the abundance of mesopelagic fishes in the CHSH diet suggests that either breeding CHSH are consuming mesopelagic fishes near the surface during the day, or that some CHSH are diving deep enough at night to capture these mesopelagic fishes during their diel migration to the surface, but these events were not captured by the tagged birds in Nimz et al. (2021).

There is some evidence for both daytime surface distribution of mesopelagic fishes and nighttime seabird foraging (of non-breeding birds). Leach's Storm Petrels (*Oceanodroma leucohoa*) have been documented feeding on a daytime swarm of Phosichthyidae in the Eastern Tropical Pacific (ETP) (Ballance & Pitman, 1999). Spear et al. (2007) observed nocturnal foraging and also documented deepwater fish (Myctophidae) in CHSH diet. In their study in the ETP, 5% of the prey items by mass obtained from seven non-breeding CHSH were attributed to nocturnal feeding (Spear et al., 2007). The breeding status of the individual birds we sampled here was unknown, so nocturnal feeding by non-breeders on deepwater fish that vertically migrate to the surface is a possibility. Regardless of when CHSH are obtaining these deep-sea fish, their impact as top predators in the marine ecosystem reaches beyond the epipelagic layer. A high prevalence of mesopelagic fishes was also detected in both wedge-tailed shearwaters in the Ogasawara Islands, Japan (Komura et al., 2018) and in Bulwer's petrels (*Bulweria bulweri*) on Selvagem Grande Island in the Madeira Archipelago, Portugal (Waap et al., 2017). These findings underscore the need for more research on the behavior of these deepwater fish species, with particular attention to juveniles, to evaluate their contribution to epipelagic food webs.

4.2 | Comparison to past studies

Metabarcoding revealed the CHSH diet is diverse, with at least 68 species (within 87 distinct taxa) and 29 families. Our methodology provides higher taxonomic resolution for CHSH diet than

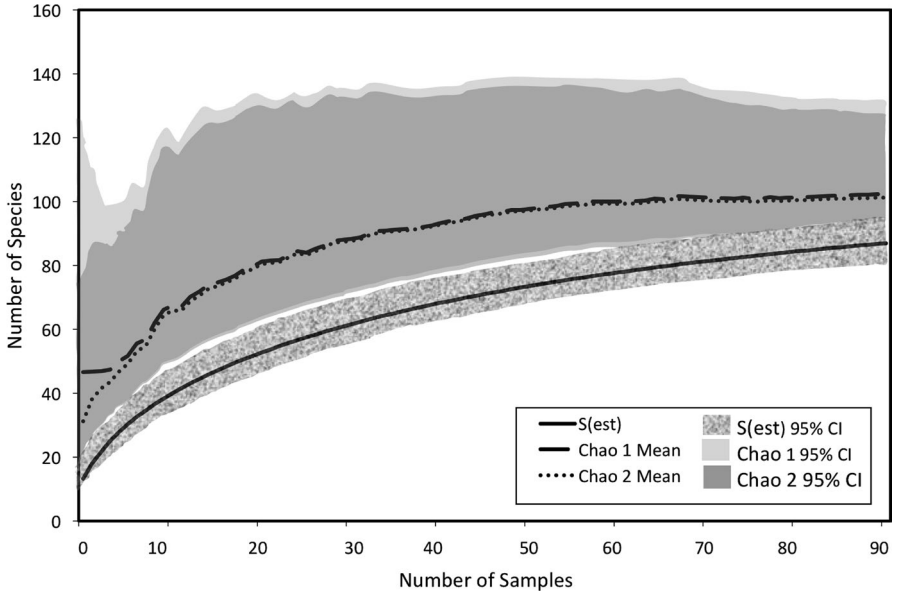

FIGURE 3 Sample-based rarefaction curves for 87 unique, identified taxa, derived from genetic analysis of the diet, showing S(est) and Chao 1 & 2 estimators. The 95% CIs are depicted with the shaded areas

previous studies, which were limited by visual methodology to family-level identification. Therefore, we compared our results to past studies at the family level. Overall, 81% percent of the families detected in the contemporary samples were previously documented in historical CHSH diets from the Northwestern Hawaiian Islands (NWHI), dating back to the 1970s (Harrison et al., 1983). The CHSH diet was consistently dominated by epipelagic species in both the 1970s and in the 2000s.

Though we documented 38% more families in the contemporary diet, we cannot discern if this result is attributed to an enhanced discovery rate due to DNA barcoding and metabarcoding or a change in CHSH diet over time. The methods Harrison et al. (1983) used to rank prey importance prevented direct comparisons to our study. Instead of calculating the frequency of occurrence of different taxa (family or genus or species), expressed as the percent of prey samples in which a particular taxonomic group occurred, they ranked their data by the lowest taxonomic level (family). Moreover, while their prey quantification combined three metrics (number, volume, and frequency of occurrence), we were only able to calculate the prey frequency of occurrence. Nevertheless, flying squid (Ommastrephidae) and flyingfish (Exocoetidae) were two of the three most prevalent families in both time periods. The Mullidae family ranked second, historically, whereas it was fourth in the contemporary diet. Conversely, the Holocentridae and Gempylidae families were much more common in the modern diet than previously documented, and were identified with both Sanger sequencing and metabarcoding approaches.

Comparisons between the historical (Harrison et al., 1983) and modern diet required additional clarification for six different families, highlighting some of the challenges in comparing diet across time and the imperative of sequenced voucher specimens. First, the Family Gonostomatidae was listed in Harrison et al. (1983) with the subtext *Vinciguerria sp*., a taxonomic group currently assigned to the family Phosichthyidae (Tomás & Panfili, 2000). Because we found a different species within Gonostomatidae in the modern CHSH diet, we included that family in our count. We also found a *Vinciguerria* species in the modern diet, but did not consider its new family name in the comparison to the historical data. Second, the genus *Macorhamphosus* (family Macorhamphosidae in Harrison et al., 1983) is currently classified by FishBase (fishbase.org; Froese & Pauly 2021) in the family Centriscidae, and is a family match to the modern diet. Otherwise, the four families found historically but missing in the modern diet (Sternoptychidae, Monacanthidae, Gonorhynchidae, Dactylopteridae) were ranked below 16 of 24 taxa, indicating they were uncommon in the historical diet.

The highly digested nature of the CHSH samples we collected was consistent with findings from previous studies of voluntary regurgitations from the NWHI (Harrison et al., 1983), and Kiritimati Island (Ashmole & Ashmole, 1967). The average number of prey items per sample was 9.4, which is 67% greater than the figure reported by Harrison et al. (1983), and similar to the 10.5 prey items per sample found by Ashmole and Ashmole (1967). By percent volume, squid was consumed in greater quantities around Kiritimati Island in the 1960s (71%; Ashmole & Ashmole, 1967) and in the NWHI in the 1970s (48%; Harrison et al., 1983) than around Hōlanikū in the 2000s (37%, measured in percent wet mass). In the contemporary diet, fish was a larger proportion (47%) of the total mass than squid (37%), and mush was also a noteworthy contributor to the total mass of the samples (16%).

Mush consists mostly of digested fish, as evidenced by the strong correlation between the mass of fish and mush mass in the regurgitation samples. Muscular flying squid mantles tend to stay intact longer through digestion, though the skin peels off and the buccal masses detach. Conversely, digested fish tissues easily slough off the skeleton and mix in the stomach. While CHSH may ingest some exoparasitic crustaceans attached to flyingfish, they are not known to forage on any other pelagic crustaceans or other taxa that would contribute to mush (Ashmole & Ashmole, 1967; Harrison et al., 1983; Spear et al., 2007). Thus, while digestion leads to the underestimation of the importance of fish in the CHSH diet, the quantification of

the mass of mush in the samples can help quantify the magnitude of this bias (Donahue et al., 2021).

Sample-based rarefaction analysis on the pooled regurgitation samples indicated that the species accumulation curve reached an asymptote within the S(est) 95% confidence intervals, indicating that we successfully characterized the contemporary diet species richness. Typically, rarefaction curves do not asymptote on large geographical scales (Gotelli & Colwell, 2011), so the broad pelagic foraging range of CHSH was represented well by our combined sampling effort over several months and years. Yet, because some of the families reported in the historical study (Harrison et al., 1983) were not found in our samples, our study likely failed to document all the possible CHSH prey.

4.3 | Temporal variability in CHSH diet

We sampled a large temporal scale, spanning three periods of the breeding season: prospecting/incubation (April), early chick-rearing (May–August), and late chick-rearing (September). Due to the opportunistic nature of the sampling, different months were sampled over five different years, between 2009 and 2017. Thus, the seasonal and interannual effects were confounded and we could not resolve significant inter- or intra-annual differences in diet. However, anecdotally, we noted a few seasonal trends for select families based on FO in the diet that warrant future study. Snake mackerel (*Gempylus serpens*) and Ommastrephid squid were highest ranked throughout all seasons of diet collection, indicating these species are a fairly consistent food source throughout the CHSH breeding season at Hōlanikū. Flyingfishes were highest ranked during the mid-summer months, which is similar to peak seasonality of this family eaten by black noddies (*Anous minutus*) at Tern Island (Seki & Harrison, 1989). Finally, the Holocentridae family ranked higher in the summer and fall than in the spring, a pattern likely indicative of their spawning seasonality (Clarke, 1991; Seki & Harrison, 1989). Moreover, previous diet studies have documented interannual variability in the reliance on fish and squid, and in the number of fish species consumed by Hawaiian seabirds (Donahue et al., 2021, Seki & Harrison, 1989).

Seasonal shifts in diet before and during the breeding season have been detected in other shearwater species (e.g., Alonso et al., 2014, Komura et al., 2018). These diet shifts are hypothesized to be driven by shifts in prey abundance, seasonal changes in prey selectivity, and/or changes in foraging effort to accommodate dietary requirements (e.g., adult vs. chick provisioning). Although information on CHSH foraging distributions is limited, their ranging behavior is likely influenced by the location and size of feeding tuna schools and the availability of juvenile fish and squid.

The ocean conditions around the NWHI during the CHSH breeding season are dynamic and influence prey abundance and distribution. Most notably, the transition zone chlorophyll front seasonally migrates over 1000 km, from lower winter latitudes of 30–35°N (February) to higher summer latitudes of 40–45°N (August) (Bograd et al., 2004). Furthermore, fish spawning is seasonal, and impacted by food availability (chlorophyll-*a*), water temperature, and ocean currents (Kobayashi & Polovina, 2006). Future studies should explore the temporal diet variability we observed, and couple diet analyses with GPS tracking of CHSH to link the prey composition with at-sea distributions of foraging individuals. Understanding these linkages will be key in predicting the trajectory of these breeding populations, given the anticipated changes to the North Pacific subtropical ecosystem (e.g., Fayet et al., 2021; Woodworth-Jefcoats et al., 2017).

4.4 | Secondary predation

We acknowledge that metabarcoding may have detected more species than CHSH consumed primarily, and that this could have also contributed to the 38% increase in documented families in the contemporary CHSH diet. We attempted to limit identifications due to secondary predation, following two approaches recommended by Hardy et al. (2017) that were feasible with our dataset.

First, based on previous studies of CHSH and the morphological identification of the prey, we assumed that fish and squid were the primary prey items, and all other matches, including Crustacea, Polychaeta, Anthozoa, Echinoidea, and Gastropoda, would likely be from secondary predation, accidental ingestion during foraging, prey parasites, or some other confounding factor like sample contamination during collection. This stringent taxonomic restriction accounted for a large majority of potential secondary prey, although larval fish consumed by piscivorous prey of CHSH could have been interpreted as a primary prey item.

Second, we used information on the prey's diet to investigate potential cases of secondary predation, by considering both co-occurrence of prey items and prey size distributions. Though previous studies indicated that CHSH eat deepwater fish (Harrison et al., 1983; Spear et al., 2007), we explored the possibility that a proportion of the mesopelagic fish families detected by metabarcoding could be secondary prey items. For example, the neon flying squid (*Ommastrephes bartramii*), a dominant CHSH primary prey item, consumes Myctophids in the central North Pacific (Watanabe et al., 2004). Myctophids were found in 89% of the diet samples that also contained Ommastrephid squid, and only occurred in two samples without squid also present. However, the CHSH sampled in this study primarily consumed juvenile squids (5–10 cm mantle length), which consume zooplankton, rather than fish at this ontogenetic stage. Therefore, we inferred that Myctophids are a primary prey of CHSH.

4.5 | Primer selection

To date, gut content analysis for seabird species using molecular approaches has primarily involved: (1) sequencing each prey item individually, which works best for fresher prey (e.g., Donahue et al., 2021), or (2) metabarcoding of feces (e.g., Deagle et al., 2007; Deagle

et al., 2009; Komura et al., 2018; Waap et al., 2017). To our knowledge, Doyle and Adams (2018) have made the only other attempt to metabarcode seabird gut contents, and they had minimal success using the 16S gene region (but see a multi-template, multi-primer approach by Oehm et al. (2017)).

Our approach was designed to successfully provide diet information for seabirds with highly digested gut contents (i.e., Doyle & Adams, 2018), using primer assays that would detect, at a minimum, both invertebrate and vertebrate prey previously documented for CHSH to the family level (Ashmole & Ashmole, 1967; Harrison et al., 1983). In our primer selection test, only the COI assays detected both fish and squid, and resolved detections to the species level for both vertebrates and invertebrates. Consistent with prior studies, the mlCOIintF/jgHCO2198 Leray primer pair outperformed the jgHCO1490/mlCOIintR pair (Leray et al., 2013). However, the BF1/BR2 primer set for COI, designed for freshwater invertebrate bioassessments (Elbrecht & Leese, 2017), was the most successful at OTU detection for our target taxa across all primer sets. The 12S and 16S assays (Evans et al., 2016) were developed to detect fishes, but two of the COI region assays resolved a higher number of OTUs matching fishes than either 12S or 16S (Table 2). Like Doyle and Adams (2018), we found the 16S assay to be one of the least successful. Only the 18S assay returned fewer OTUs after applying the match thresholds (Table 2). The 18s assay did not resolve vertebrates to the species level, nor did it detect our invertebrate targets (squid). Our primer experiment may be used to guide primer choices for future dDNA diet studies of pelagic seabirds, with recognition that new primer sets arise quickly, and successful primer assays depend on primer binding efficiency and the availability of prey sequence data. For example, since the initiation of our study, the Miya et al. (2015) primer set has become widely adopted for fish metabarcoding, and will likely provide increased taxonomic coverage over the AM12s assay used here. Similarly, a recently published 18s primer set targeting Cephalopoda may also improve results over the COI assays used here (de Jonge et al., 2021). We encourage comparative primer set trials and use of multiple optimal primer sets in each metabarcoding study when possible.

4.6 | Caveats and recommendations for the future of metaBARFcoding

The high throughput metabarcoding approach has improved our knowledge of CHSH diets by detecting additional prey species from more degraded samples than morphology or Sanger sequencing alone. However, sorting and characterizing the diet classes morphologically (e.g., fish, squid) added details on the numbers and biomass of prey items that are not obtainable from sequence data, making the morphological and metabarcoding methodologies complementary. Each of these complementary methods also addresses a different diet timescale, with fresh, whole prey that can be identified morphologically as the most recent, followed by degraded, but DNA barcoded prey, dDNA metabarcoded prey, and hard parts such as squid beaks and otoliths that may remain over much longer time periods (Corse et al., 2015); Himmelsbach, 2021). With the increasing adoption of one or more of these complementary approaches, narrowing the timescales for each prey digestion state will lead to a more integrative understanding of seabird diets (e.g., Oehm et al., 2017). We provide the following additional recommendations for future metaBARFcoding efforts.

4.6.1 | Comparing metabarcoding with Sanger sequence barcoding

The metabarcoding method worked well to characterize the diversity of the contemporary CHSH diet, but required a relatively large number of samples to characterize total prey richness (Figure 3). Future pelagic seabird diet studies should plan on similar sample sizes to characterize the rare prey in seabird diets at the spatial or temporal scale of interest (e.g., individuals, populations, regions, breeding seasons, years). The Sanger-sequenced prey items that were not detected in the metabarcode data from the same regurgitation may be due to the removal of whole prey items prior to pelleting tissue in the RO water. The individual prey items were components of another study, so grinding items to homogenize the entire sample was not feasible in our situation. Prior studies evaluating eDNA metabarcoding with Sanger sequencing similarly did not always detect 100% of the Sanger-sequenced species within their metabarcode data (e.g., Kelly et al., 2014). These false negatives are often due to uneven DNA primer efficiency across taxa, especially when prey biomass is skewed in an individual predator's gut contents (Elbrecht et al., 2017). Furthermore, taxa with poor DNA primer-template affinity (e.g., Elbrecht & Leese, 2017) can evade detection even when they are a subset of the taxa specifically targeted (e.g., Timmers et al., 2020). To mitigate these false negatives, we recommend future studies follow a one-locus, several-primer approach to metabarcoding when feasible (e.g., Corse et al., 2019). Authors should also consider combining the barcoding and metabarcoding approaches, particularly if individual diet resolution is required. Specifically, we recommend some combination of: (1) processing multiple subsamples per regurgitation to metabarcode, (2) amplifying those subsamples at multiple PCR priming sites per DNA assay, (3) increasing the frequency of DNA barcode spot checking, and, (4) assuming that only presence/absence is being analyzed, adding small tissue subsamples from whole prey to the pelleted tissue, since these whole individuals may not have contributed tissue to the metabarcoded "mush."

4.6.2 | Field collection protocols

We recommend future seabird metabarcoding studies store the regurgitated material in 95%–100% non-denatured ethanol rather than freezing in RO water. This approach does not require a freezer to preserve the samples, would maintain the structure of the regurgitation, and would expedite the pelleting and extraction process.

Mitigating contamination during sample collection and processing is critical in genetic studies. The study site was coral substrate commonly accessed by a variety of seabirds, and trace amounts of fecal or other contamination from a variety of organisms could have contacted regurgitated prey during the collection process. To minimize contamination during sample collection, seabird regurgitations should be captured prior to contact with the ground. For example, a liner or hood could be placed under the bird after ground-netting and before handling. If smaller pelagic seabirds are captured by mist net, setting a large plastic liner under the net span would create a barrier between the ground and regurgitations.

Additionally, field controls were not included in our study because we did not anticipate using these samples for metabarcoding; however, we highly recommend using field controls (e.g., filling tubes with RO water in the field, sampling the substrate where regurgitations are collected) and processing these alongside the other regurgitation samples to account for any contamination during sampling.

4.6.3 | Lab controls

We recommend using mock community samples of species that do not occur in the foraging range of the study organism to account for any lab contamination. Although there was minimal cross-contamination in our study, using a mock community sample, as opposed to a negative DNA extraction and PCR control (e.g., extracting RO water) enabled the level of contamination to be measured, and an appropriate filter applied to the sequencing results. We recommend additionally including negative controls for all DNA extraction and PCR steps so that any contamination can be identified as soon as it occurs, even though the mock community has the added benefit of enabling contamination quantification (but post-sequencing). Finally, if potential prey are known and obtainable, an additional mock community with known quantities of common prey would be a beneficial positive control.

5 | CONCLUSION

Without a molecular approach, particularly metabarcoding, it would have been impossible to describe the CHSH diet with high taxonomic resolution due to the degraded prey state. The complementary approaches of DNA detection for species-level and family-level prey identification and morphological sorting and characterizing of diet classes for prey enumeration and weight provided insights into the foraging ecology of this generalist predator that could not be obtained from a single methodology. Altogether, our results underscore the use of "metaBARFcoding" as a robust approach to characterize degraded prey samples from seabirds.

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CONFLICT OF INTERESTS

The authors have no conflict of interest to disclose.

AUTHOR CONTRIBUTIONS

KDH and IN conceived the idea of using "metaBARFcoding" to identify highly degraded prey items and contributed to study design. MAR designed the genetics methods, with extensive laboratory assistance from JB, and some assistance from IN and MI. IN, KDH and MI contributed to data analysis and interpretation. IN wrote the manuscript, with methods by MAR and significant contributions by MI and KDH. CAV facilitated sample collection on Kure Atoll. All authors contributed to editing and revising the drafts.

DATA AVAILABILITY STATEMENT

Raw Illumina sequence files have been deposited in the NCBI Sequence Read Archive (SRA) under BioProject accession numbers PRJNA774371 (initial primer testing) and PRJNA774425 (diet study). The associated metadata for the DNA sequence barcodes and SRA sequence files are located in the Genomic Observatories MetaDatabase (GEOME; Deck et al., 2017) at https://geome-db.org/workbench/project-overview?projectId=440. All other data for this study can be found in the Dryad Digital Repository (https://doi.org/10.5061/dryad.d51c5b04m).

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