## Electronic Supplementary Materials – Quantifying a wildlife conflict: estimates of seabird predation by recovering fur seals across southeastern Australia

## S1 Methods

*S1.1 Sample collections and site background information*

Long-nosed fur seal pups have been recorded (to the best of our knowledge) at Deen Maar Island since 2002, Cape Bridgewater since 2008, Gabo Island since 2016, and Barunguba since 2000 [(Arnould et al., 2003;](https://www.zotero.org/google-docs/?QBhosL) McIntosh et al. unpub. data; [Shaughnessy et al., 2001)](https://www.zotero.org/google-docs/?5lHpvH). Pup numbers, as an index of population size in 2013, were ~100 at Cape Bridgewater, ~ 24 at Deen Maar Island (and in 2017), 8 at Gabo Island and ~42 at Barunguba [(McIntosh et al., 2014;](https://www.zotero.org/google-docs/?Rgkcns) McIntosh et al. unpublished data[)](https://www.zotero.org/google-docs/?HlG32e) (Fig. 1).

Additionally, Phillip Island, in north-central Bass Strait, is home to the largest little penguin colony, with an estimated 31,000 breeding pairs of penguins in 2010 [(Sutherland & Dann, 2014)](https://www.zotero.org/google-docs/?3e5AQ8). The success of this colony is largely attributed to fox eradication and habitat restoration [(Rout et al., 2014; Sutherland & Dann, 2014)](https://www.zotero.org/google-docs/?XUwBYO). The internationally popular Penguin Parade, managed by Phillip Island Nature Parks (The Nature Parks), provided little penguin viewing experiences for over 757,846 visitors in 2019-20 [(Phillip Island Nature Parks, 2020)](https://www.zotero.org/google-docs/?qItaWv).

*S1.2 Morphological identification of seabird remains in long-nosed fur seal scats*

Little penguin feathers are recognisable by their blue tinge and compact shape (Fig. 2d). Shearwater feathers are long, tapered and grey in colour. Seabird carcasses collected at Phillip Island were used to verify feather identity. The presence of bird hard parts in a single scat was recorded as one bird unless multiple appendages or heads suggested otherwise.

### S1.2 DNA metabarcoding of seabird genetic material & reaction conditions

DNA was extracted from 250 mg of faecal subsamples using MoBio PowerSoil® DNA Isolation Kits ([www.mobio.com](http://www.mobio.com)) with modifications to the manufacturer’s instructions made in response to the extraction optimisation described herein. These included an overnight digestion phase in cell lysis buffer (C2 solution) at 4˚C, and the incubation step in inhibitor removal solution was extended from 5 to 60 minutes at 4˚C. The eluted DNA was stored at -20˚C. DNA was extracted from a domestic chicken (*Gallus gallus domesticus*), and one of the target taxa, a little penguin (*Eudyptula minor*) carcass obtained by Phillip Island Nature Parks, and both were used as both positive controls and to test primer specificity. Extraction was from muscle tissue, from the centre of the birds’ tissue matrix (25mg), using Bioline Isolate II Genomic DNA Kits (<https://www.bioline.com/us/>) as per manufacturer instructions. The DNA was stored in multiple aliquots to avoid freeze/thawing effects. A dedicated controlled eDNA laboratory was used at RMIT University, Bundoora, Victoria, with separate spaces and rooms designated for the physical separation of eDNA extraction, pre-PCR preparations and post-PCR procedures. Positive and negative controls (extraction and PCR) were used to identify potential contamination at each laboratory procedural step from DNA extraction to diagnostic PCR steps.

Two diagnostic endpoint PCR (dPCR) assays were tested on a subset of sample extracts (n =10, at both neat and 1:10 concentrations) and positive controls (chicken and penguin) using two primers. The Bird12sa/h primer pair targets the 12S ribosomal RNA (rRNA) gene [(Cooper, 1994)](https://www.zotero.org/google-docs/?m18GNS), and the AWCF1/R6 primer pair targets the cytochrome c oxidase 1 gene (COI; Table S1) [(Patel et al., 2010)](https://www.zotero.org/google-docs/?0wHblZ). Diagnostic dPCRs were run on Bio Rad C1000 Touch thermal cycler using cycling steps outlined in Table S2. We used the AmpliTaq Gold® 360 Master Mix using reagents and concentrations provided by the manufacturers (Table S2). Both primers were tested using positive controls and run on a gradient PCR from 52–58˚C (with 1˚C increments) to optimise the annealing temperature. Amplification was successful and similar at each temperature on that gradient. Ultimately, we selected 57˚C for the Bird12sa/h primer [(Cooper, 1994)](https://www.zotero.org/google-docs/?yzxh9M), and 54˚C for AWCF1/R6 [(Patel et al., 2010)](https://www.zotero.org/google-docs/?0qzMhy). Diagnostic endpoint PCR products were run on 1.5% agarose gels to determine the presence/absence of amplified target bird DNA. While the COI assay successfully amplified penguin, and more so chicken, it detected bird DNA in < 5% of the subset of samples. The shorter Bird12sa/h assay was found to be more sensitive than COI. Given the degraded nature of DNA found in faecal samples this was expected. Consequently, due to time constraints and costs, we chose to continue with only the Bird 12sah assay.

The Ramaciotti Centre for Genomics (RCG) laboratories, at the University of NSW, Sydney performed the library build and sequencing. Samples were assigned a unique MID (Multiplex IDentifier) tag combination, next generation sequencing (NGS) adaptors and the Bird12sa/h primer using single-step fusion tag PCR. The amplicons were purified and blended in equimolar concentrations to form a library, which was sequenced with a 150 bp paired-end sequencing kit (Illumina Miseq v2 Nano 150 bp). We chose to use the single-step fusion PCR procedure over the cheaper and faster two-step PCR approach because cross contamination of amplified DNA can occur among samples within the initial diagnostic step of a two-step PCR. That cross-contamination, however minor, can become significant during the second amplification step where MID tags are assigned; and where there is the potential for the tags to be paired and amplified with the wrong sample. This type of error is difficult to detect and risks cross-contamination of amplified DNA among samples between initial PCR products and terminal PCR products.

### S1.3 Bioinformatics pipeline and sequence quality filtering

The demultiplexed sequence reads were assigned to the correct sample using the unique MID tag combinations. Pairs of forward and reverse target sequences were then stitched in Geneious (overlap = 70 bp). The MID tags, NGS adaptor sequences and the Bird12sa/h forward and reverse primers, were subsequently trimmed using Geneious R8.1.5 [(Kearse et al., 2012)](https://www.zotero.org/google-docs/?9vw497). Sequences were discarded if they did not contain exact matches to both the forward and reverse PCR primers, tags and adaptor sequences; failed to pair; or, were shorter than the primer product length (< 200 bp). Discarded sequences typically corresponded to low-quality reads or primer dimer.

Target sequences were merged and clustered into molecular operational taxonomic units (OTUs) using the *UPARSE* algorithm (*USEARCH*) [(Edgar, 2010; Edgar & Flyvbjerg, 2015)](https://www.zotero.org/google-docs/?0qVf5D). This filtering process: (i) removes chimeric sequences; (ii) low abundance sequences are discarded below expected threshold abundances accounting for sequencing platform error (threshold value: < 1% of total number of unique sequences); (iii) and sequences are clustered using a 97% similarity criterion [(similar to Berry et al., 2017; Hardy et al., 2017)](https://www.zotero.org/google-docs/?rsnk2w). Discarding sequence clusters containing <1% of the total number of unique sequences removes low abundance sequences thereby minimising the risk of erroneous sequences and false positives from sequencing error, and vastly improves confidence in the analysis of the remaining sequences [(Berry et al., 2017)](https://www.zotero.org/google-docs/?Tj1YqF). Thus a total of 7370 unfiltered unique seabird DNA sequences were parsed to the standard QC and OTU analysis pipeline, using a threshold value of 73 (1% of unique sequences) as the minimum cluster size. This is a conservative threshold to account for any sequencing platform bias [(Berry et al., 2017)](https://www.zotero.org/google-docs/?0aJP9e). This effectively filtered over 64,700 down to 35,353 individual seabird DNA sequences across all 99 samples.

Consensus sequences for each OTU (Table S3) were queried against the National Center for Biotechnology Information’s (NCBI) GenBank nucleotide database using BLASTn (Basic Local Alignment Search Tool) [(Altschul et al., 1990; Benson et al., 2005)](https://www.zotero.org/google-docs/?zj6V7O). The results were then visualised in MEGAN (MEtaGenome ANalyser) [(Huson et al., 2007)](https://www.zotero.org/google-docs/?O0ZYt0). Assignments to taxa were made following criteria and taxonomic reference databases outlined in [Hardy et al. (2017)](https://www.zotero.org/google-docs/?K0QSwc) and [Deagle et al. (2009)](https://www.zotero.org/google-docs/?gWKhfo). The objective of these criteria was to further ensure maximum confidence in making a taxonomic identification, and minimise the risk of false positives. Potential prey identifications were individually investigated by consulting reference resources to assess their likelihood. The factors considered include: (1) ensuring that the prey’s geographic distribution broadly matched that of the expected southeast Australian foraging areas of the long-nosed fur seals, and (2) checking the diversity of closely related species and the presence/absence of voucher sequences for these in GenBank to ensure that any other likely prey species were not overlooked for want of genetic reference information. A range of reference databases were consulted, including: Atlas of Living Australia [(ALA, 2019)](https://www.zotero.org/google-docs/?aFx4Ts), the [Australian Museum (2019)](https://www.zotero.org/google-docs/?lPTfxM) reference base and [Redmap (2019)](https://www.zotero.org/google-docs/?ujGeUY). All the identified seabirds occurred within the geographic ranges of the LNFS and are considered viable prey species for LNFS.

**Table S1.** Details for primers used, target taxa and gene, as well as designer references.

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| PCR Assay | Primer set used | Target Taxa | Gene | Primer sequence 5' to 3' | Amplicon length (base pairs) | Reference | Assay Temp (˚C) |
| Bird12S | 12Sa (F) | Birds | 12S rRNA | CTGGGATTAGATACCCCACTAT | ~230 bp | Cooper (1994) | 57˚ |
|  | 12Sh (R) |  |  | CCTTGACCTGTCTTGTTAGC |  |  |  |
| BirdCOI | AWCF1 (F) | Birds | COI | CGCYTWAACAYTCYGCCATCTTACC | ~848 bp | Patel et al. (2010) | 54–55˚ |
|  | AWCR6 (R) |  |  | ATTCCTATGTAGCCGAATGGTTCTTT | |  |  |

**Table S2.** PCR reaction reagent concentrations and conditions.

|  |  |  |
| --- | --- | --- |
| *Reagents/Concentration* | Cooper (1994) | Patel et al. (2010) |
| **Primer:** | **Bird12sa/h** | **AWCF1/R6** |
|  | 1x (µL) | 1x (µL) |
| Molecular grade H2O | 8.5 | 6.5 |
| AmpliTaq Gold® | 12.5 | 12.5 |
| F primer (10 µM) | 1 | 2 |
| R primer (10 µM) | 1 | 2 |
| Template | 2 | 2 |
| Total master-mix | 25 | 25 |
| *PCR cycling conditions* |  |  |
| Denaturing step: | 95˚C for 5 min | 95˚C for 5 min |
| Primer annealing | 95˚C for 30s, 57˚C for 30s and 72°C for 45s | 95˚C for 30s, 54˚C for 30s and 72°C for 45s |
| Number of cycles | 50 | 50 |
| Final extension | 10 min final extension of 72˚C | 10 min final extension of 72˚C |

## S2. Results supplement

**Table S3.** Generalised linear model with binomial distribution for a) seabird and b) little penguin detections in long-nosed fur seal scats across sampling group (combined location and sampling time) for seabird diagnostic morphological remains or genetic material (significant variables in **bold**,P > 0.05).

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| **Model** | **Data type** | **Explanatory variables** | **df** | **Residual deviance** | **Residual df** | **Deviance** | **P** |
| Seabird | Detection (yes/no) | Method | 1 | 0.2081 | 98 | 114.403 | 0.6483 |
|  | *binomial* | Group | 6 | 15.4554 | 92 | 98.948 | **0.0170 \*** |
| Penguin | Detection (yes/no) | Method | 1 | 1.6403 | 98 | 95.604 | 0.2003 |
|  | *binomial* | Group | 6 | 8.1254 | 92 | 87.479 | 0.2291 |

**Table S4.** Genetic blast identifications of operational taxonomic units (OTUs), including information on query length and coverage, accession number and consensus sequence for the identification.

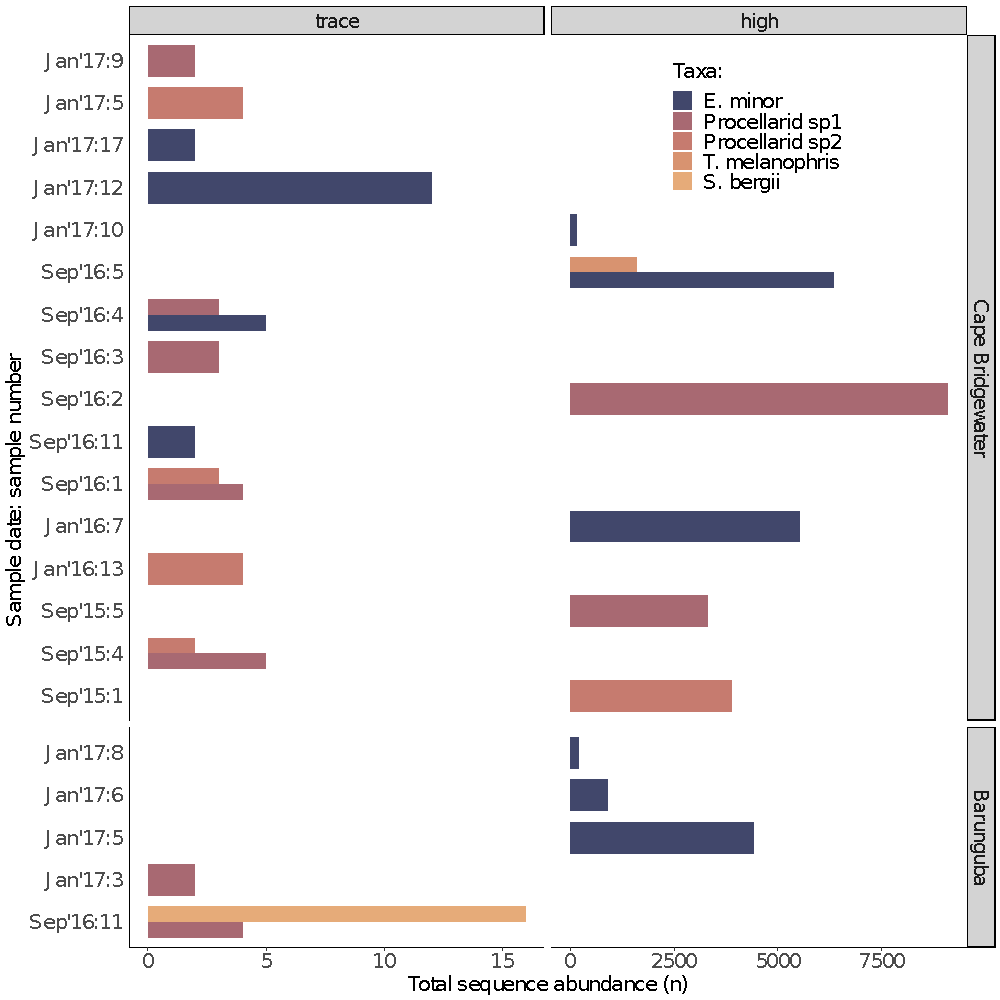
|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **OTU # (sequence abundance)** | **Query base-pair length (coverage)** | **ID (classification)** | **Representative taxon (accession number)** | **Consensus sequence** |
| OTU\_1 (17,558) | 231 (100%) | *Eudyptula minor* (species) | *Eudyptula minor* (MF370525) | GCCTAGCCCTAAATCTTGATACTTTCTATCACCAAAGTATCCGCCTGAGAACTACGAGCACAAACGCTTAAAACTCTAAGGACTTGGCGGTGCCCCAAACCCACCTAGAGGAGCCTGTTCTATAATCGATAACCCACGATGCACCCAACCACTCCTTGCCAAAACAGCCTATATACCGCCGTCGCCAGCCCACCTCCCCTGAGAGCCTAACAGTGAGCCTAATAGCCCTCC |
| OTU\_2 (12,413) | 256 (95–97%) | Procellariidae sp.1 (family) | *Pterodroma brevirostris* (AY158678) | CTGGGATTAGATACCCCACTATGCTTAGCCCTAAATCTTGATACTTACCCTACTGAAGTATCCGCCTGAGAACTACGAGCACAAACGCTTAAAACTCTAAGGACTTGGCGGTGCCCCAAACCCACCTAGAGGAGCCTGTTCTATAATCGATAACCCACGATACACCCGACCACTCCTTGCCGAAGCAGCCTACATACCGCCGTCGCCAGCTCACCTTTC-TGAAAGCACAGCAGTGAGCACAATAGCAACCAACATC |
| OTU\_3 (3,902) | 257 (95–97%) | Procellariidae sp.2 (family) | *Puffinus* sp. (AF173572) | GCTTAGCCCTAAATCTTGATACTTACCTTACTGAAGTATCCGCCTGAGAACTACGAGCACAAACGCTTAAAACTCTAAGGACTTGGCGGTGCTCCAAACCCACCTAGAGGAGCCTGTTCTATAATCGATAACCCACGATAAACCCAACCGCTCTTTGCCAAAGCAGCCTACATACCGCCGTCGCCAGCTCACCTTTCCTGAAAGTATAACAGTGAGCACAATAGCCCCCAGCATC |
| OTU\_4 (1,598) | 254 (100%) | *Thalassarche melanophris* (species) | *Thalassarche melanophris* (AY158677) | CTGGGATTAGATACCCCACTATGCCTAGCCCTAAATCTTGATACTTACCCCACCAAAGTATCCGCCCGAGAACTACGAGCGCAAACGCTTAAAACTCTAAGGACTTGGCGGTGTCCCAAATCCACCTAGAGGAGCCTGTTCTATAATCGATAACCCACGATACACCCAACCGTTCCTTGCCAAAACAGCCTACATACCGCCGTCGCCAGCCCACCTCCCCTGAGAGCTCAACAGTGGACACAATAGCCTACCCC |
| OTU\_5 (16) | 232 (100%) | *Sterna bergii* (species) | *Sterna bergii* (MH006905.1) | GCCTAGCCCTAAATCTTGATGTTTGTCATACTAAAGCATCCGCCTGAGAACTACGAGCACAAACGCTTAAAACTCTAAGG ACTTGGCGGTGCCCCAAACCCACCTAGAGGAGCCTGTTCTGTAATCGATAACCCACGATTTACCCGACCACCCCTTGCTA AGGCAGCCTACATACCGCCGTCGCCAGCTCACCTTCACTGAGAGCCAAACAGTGAGCGCAATAGCTCAACTC |

**Table S5.** Individual sample composition and OTU analysis output table.

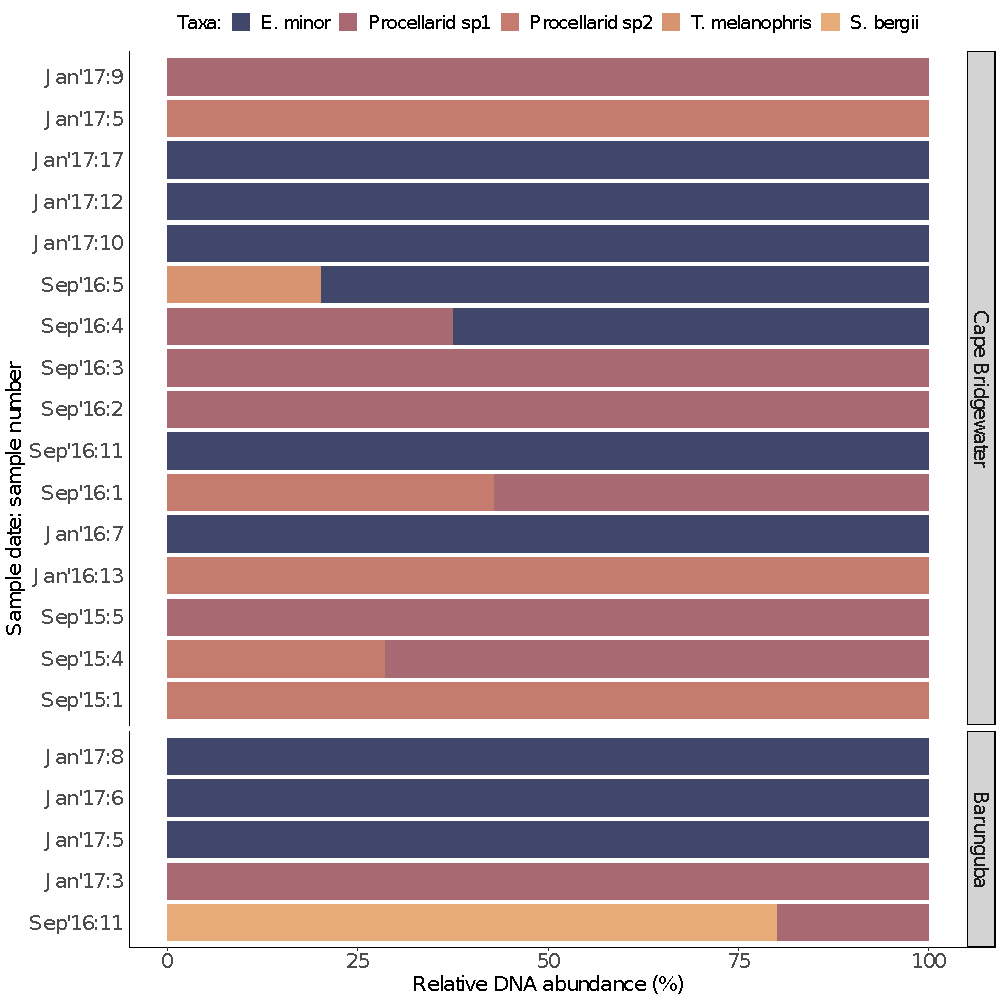
|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Prey ID** | ***Eudyptula minor (MF370525)*** | **Procellariidae sp.1 (family)** | **Procellariidae sp.2 (family)** | ***Thalassarche melanophris (AY158677)*** | ***Sterna bergii* (MH006905.1)** |
| **OTU ID** | **OTU\_1** | **OTU\_2** | **OTU\_3** | **OTU\_4** | **OTU\_5** |
| CBW1\_16\_7\_S78\_Bird12sB | 5520 | 0 | 0 | 0 | 0 |
| CBW1\_16\_13\_S81\_Bird12sB | 0 | 0 | 4 | 0 | 0 |
| CBW1\_17\_5\_S91\_Bird12sB | 0 | 0 | 4 | 0 | 0 |
| CBW1\_17\_9\_S92\_Bird12sB | 0 | 2 | 0 | 0 | 0 |
| CBW1\_17\_10\_S93\_Bird12sB | 159 | 0 | 0 | 0 | 0 |
| CBW1\_17\_12\_S94\_Bird12sB | 12 | 0 | 0 | 0 | 0 |
| CBW1\_17\_17\_S95\_Bird12sB | 2 | 0 | 0 | 0 | 0 |
| CBW9\_15\_1\_S75\_Bird12sB | 0 | 0 | 3890 | 0 | 0 |
| CBW9\_15\_4\_S76\_Bird12sB | 0 | 5 | 2 | 0 | 0 |
| CBW9\_15\_5\_S77\_Bird12sB | 0 | 3302 | 0 | 0 | 0 |
| CBW9\_16\_1\_S83\_Bird12sB | 0 | 4 | 3 | 0 | 0 |
| CBW9\_16\_2\_S84\_Bird12sB | 0 | 9094 | 0 | 0 | 0 |
| CBW9\_16\_3\_S85\_Bird12sB | 0 | 3 | 0 | 0 | 0 |
| CBW9\_16\_4\_S86\_Bird12sB | 5 | 3 | 0 | 0 | 0 |
| CBW9\_16\_5\_S87\_Bird12sB | 6348 | 0 | 0 | 1599 | 0 |
| CBW9\_16\_11\_S90\_Bird12sB | 2 | 0 | 0 | 0 | 0 |
| MI9\_16\_11\_S100\_Bird12sB | 0 | 4 | 0 | 0 | 16 |
| MI1\_17\_5\_S102\_Bird12sB | 4415 | 0 | 0 | 0 | 0 |
| MI1\_17\_6\_S103\_Bird12sB | 894 | 0 | 0 | 0 | 0 |
| MI1\_17\_8\_S104\_Bird12sB | 206 | 0 | 0 | 0 | 0 |
| Total sequence abundance | 17558 | 12413 | 3902 | 1598 | 16 |
| Total of samples containing each seabird taxon (n) | 10 | 8 | 5 | 1 | 1 |

**Table S6.** Haplotype abundance from each sample. From the six samples containing abundant little penguin DNA (OTU1), the nine most abundant sequences were extracted for haplotype analysis.

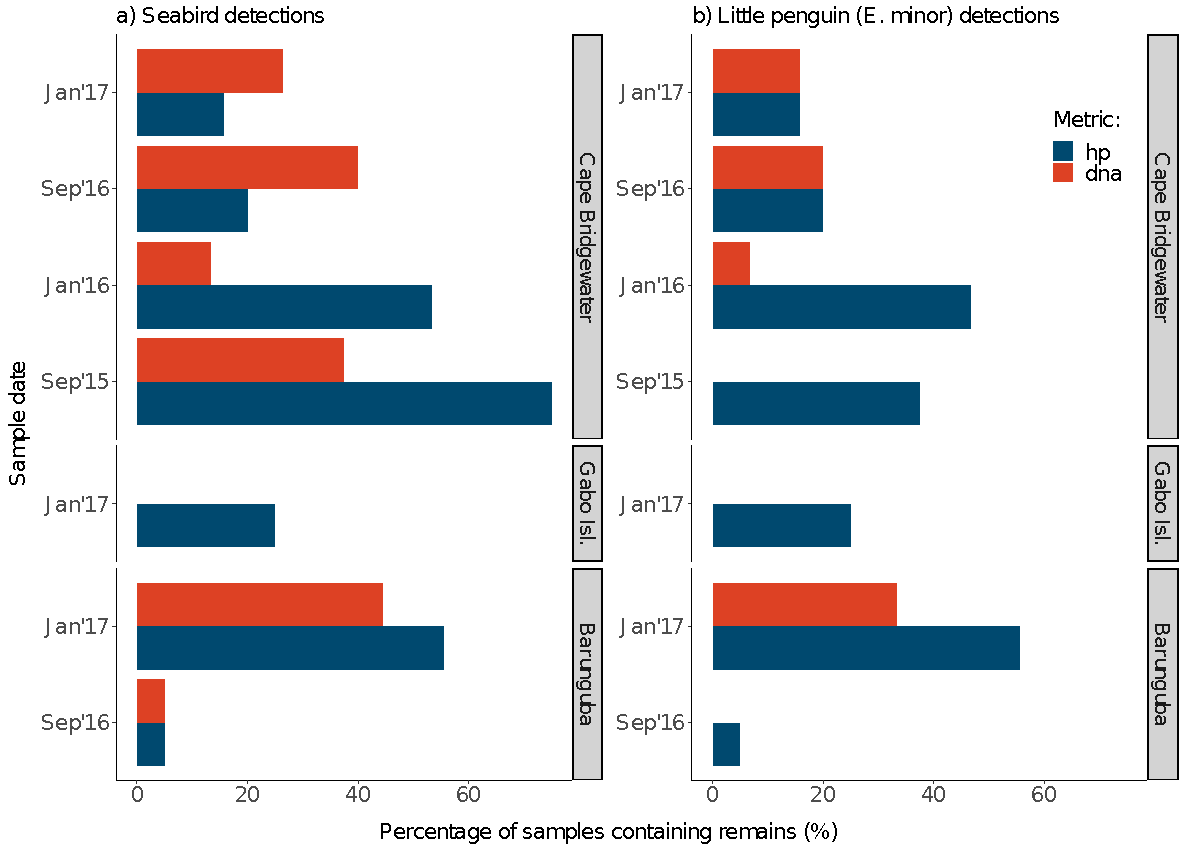
|  |  |  |  |
| --- | --- | --- | --- |
| **Sample ID**  **(total number of little penguin sequences)** | **Sequence a**  **(haplotype)** | **Sequence b**  **(haplotype)** | **Sequence c**  **(haplotype)** |
| CBW1\_16\_7\_S78\_Bird12sB (5520) | 5315 (1) |  |  |
| CBW1\_17\_10\_S93\_Bird12sB (159) | 151 (4) |  |  |
| CBW9\_16\_5\_S87\_Bird12sB (6348) | 5210 (1) | 454 (5) | 400 (4) |
| MI1\_17\_5\_S102\_Bird12sB (4415) | 4289 (1) |  |  |
| MI1\_17\_6\_S103\_Bird12sB (894) | 589 (1) | 262 (2) |  |
| MI1\_17\_8\_S104\_Bird12sB (206) | 200 (1) |  |  |

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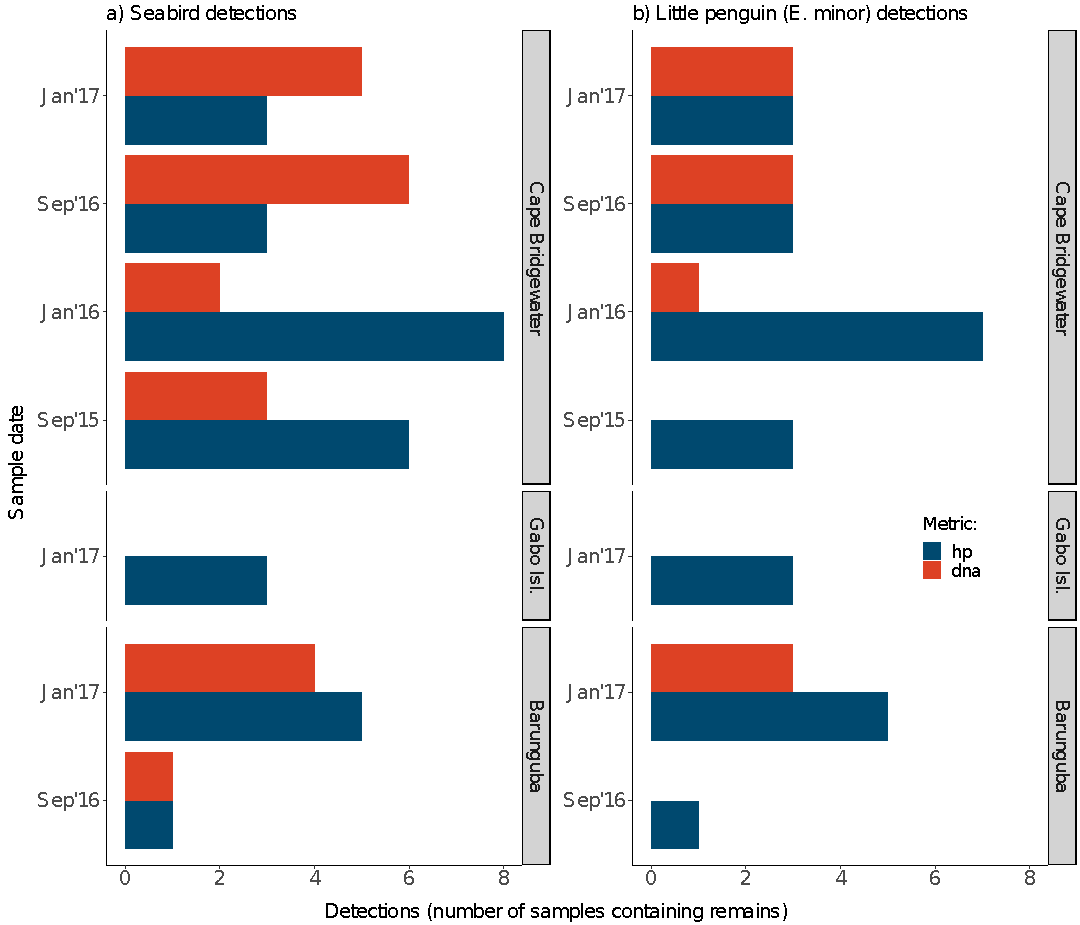
**Figure S1.** Total abundance of DNA sequences obtained within samples (n = 99), for all five seabird taxa and after sequence quality filtering procedures described in section S1.3.

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**Figure S2.** Relative genetic contribution within seabird-positive LNFS samples (n = 21) of seabird taxa identified and illustrated as a proportion of DNA abundance recovered for each taxon within samples, across locations and sampling time.



**Figure S3.** Percentage of samples detecting seabirds by method (hp = hard-part analysis, dna = DNA metabarcoding), location, and sampling time, for a) all seabird detections and b) little penguin (*E. minor*) detections, at Cape Bridgewater, Gabo Island and Barunguba.

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**Figure S4.** Numerical detections of seabirds by method (hp = hard-part analysis, dna = DNA metabarcoding), location, and sample date (month’ year), for a) all seabird and b) little penguins (*E. minor*).

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