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

## S1 Methods

### S1.1 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 unpublished data[)](https://www.zotero.org/google-docs/?HlG32e) (Fig. 1).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). Seabird morphological remains are conspicuous across long-nosed fur seal colonies in southeastern Australia (Fig. 2).

### S1.2 Additional information on morphological identification of seabird remains in long-nosed fur seal scats

All prey items were identified from hard-parts using methods described by Page et al. (2005). 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.

### S1.3 Information on multiple primers pilot study

Two diagnostic endpoint PCR (dPCR) assays were tested using two avian specific primers (Table S1). 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) [(Patel et al., 2010)](https://www.zotero.org/google-docs/?0wHblZ) (Table S1). 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 (chicken and penguin) 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. 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), and further tested amplification success in both primers on a subset of our faecal sample extracts (n =10, at both neat and 1:10 concentrations). 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, but more so chicken in positive controls, it only detected bird eDNA in < 5% of our samples. The Bird12sa/h assay targets a shorter gene fragment and is therefore more sensitive than the COI primer targeting a long fragment eDNA. Given the degraded nature of DNA found in faecal samples, it was expected that the assay targeting shorter amplicon lengths (~230 bp, Bird12sa/h) would produce a greater number of positive results, compared to the assay targeting long amplicon lengths (~850 bp, COI). Additionally, the Bird12sa/h primer produced a similar proportion of positive results for seabird eDNA to the overall detection rates of diagnostic seabird hard-parts in samples (~30%). Consequently, due to time constraints and costs, we chose to continue with only the Bird 12sa/h assay and discuss the availability and use of multiple specific genetic assaying tools in avian eDNA.

### S1.4 Bioinformatics pipeline and sequence quality filtering

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 thus individually investigated by consulting reference resources to assess their likelihood of consumption. 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.** Primers used, target taxa and genes, 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. Additional data and figures

**Table S3.** Genetic BLASTn (Basic Local Alignment Search Tool) identifications of operational taxonomic units (OTUs), including information on query length and coverage, accession number and consensus sequence for the identification (Benson et al., 2005).

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **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 S4.** Individual sample composition obtained from quality filtered sequences which have been clustered into molecular operational taxonomic units (OTUs). Here low abundance clusters across samples were deleted, using a threshold of < 1% (n < 74) of the total abundance of unique sequences in this study (n = 7,370). This resulted in 35,498 total filtered sequences, 26 unique (sequences because thousands were combined to within 97% similarity), 5 OTU’s, for 25 samples. We then mapped back filtered sequences to existing OTUs. Thus allowing for individual samples that contain very low to trace amounts of target sequences of high quality.

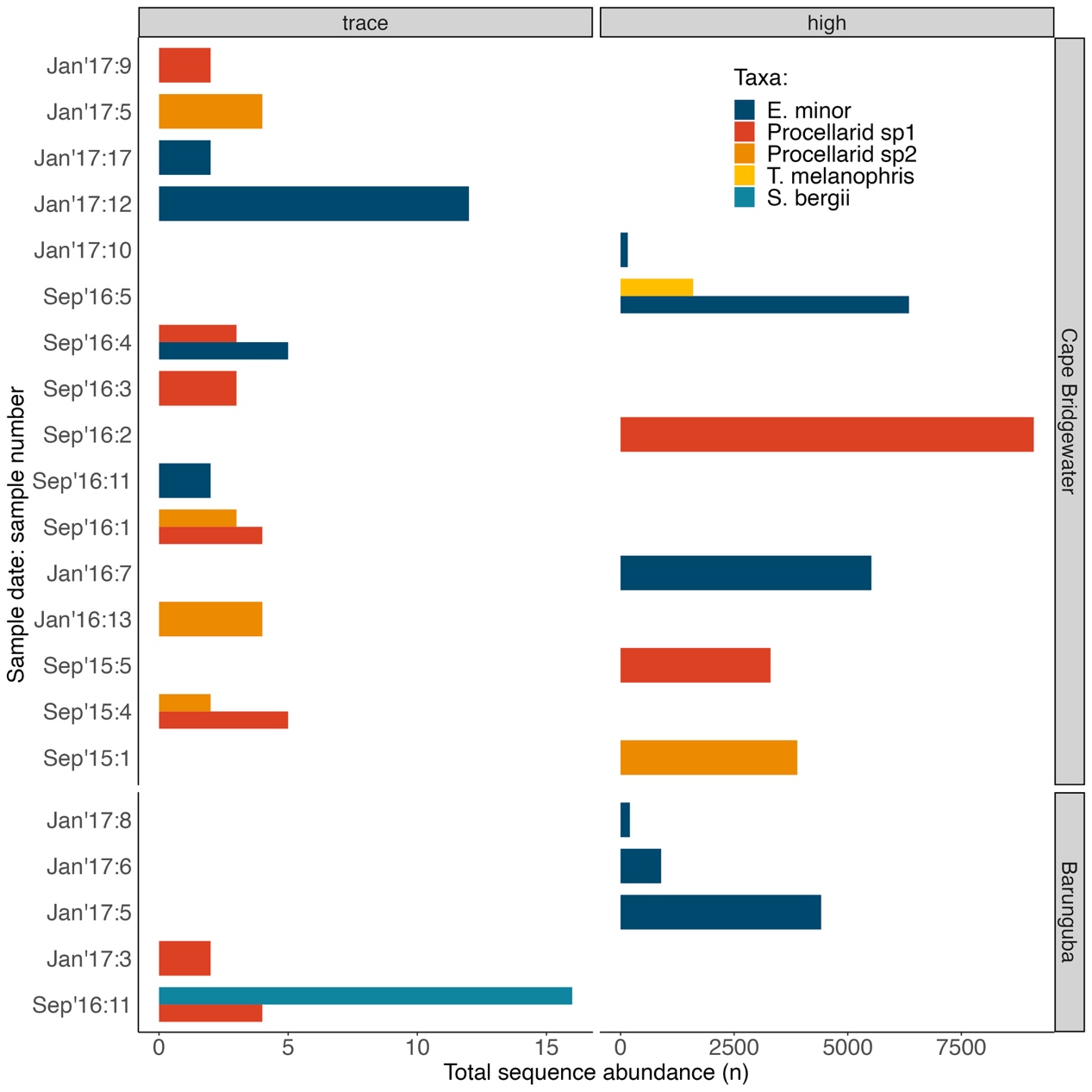
|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Prey ID** | ***Eudyptula minor (MF370525)*** | **Procellariidae sp.1 (family)** | **Procellariidae sp.2 (family)** | ***Thalassarche melanophris (AY158677)*** | ***Sterna bergii* (MH006905.1)** |
| **OTU ID** | **OTU\_1** | **OTU\_2** | **OTU\_3** | **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 | 17563 | 12417 | 3903 | 1599 | 16 |
| Total of samples containing each seabird taxon (n) | 10 | 8 | 5 | 1 | 1 |

**Table S5.** For each sample containing abundant little penguin DNA (OTU 1, n = 6), we selected the most abundant unique sequences. Note that only where there was a large sequence abundance within the sample, and where there was more than one base-pair difference in the sequences for secondary clusters of unique sequences, were a second and/or third sequence used for the haplotypes from the samples. The sequence abundance is included below for the whole sample and the haplotypes selected for further analysis.

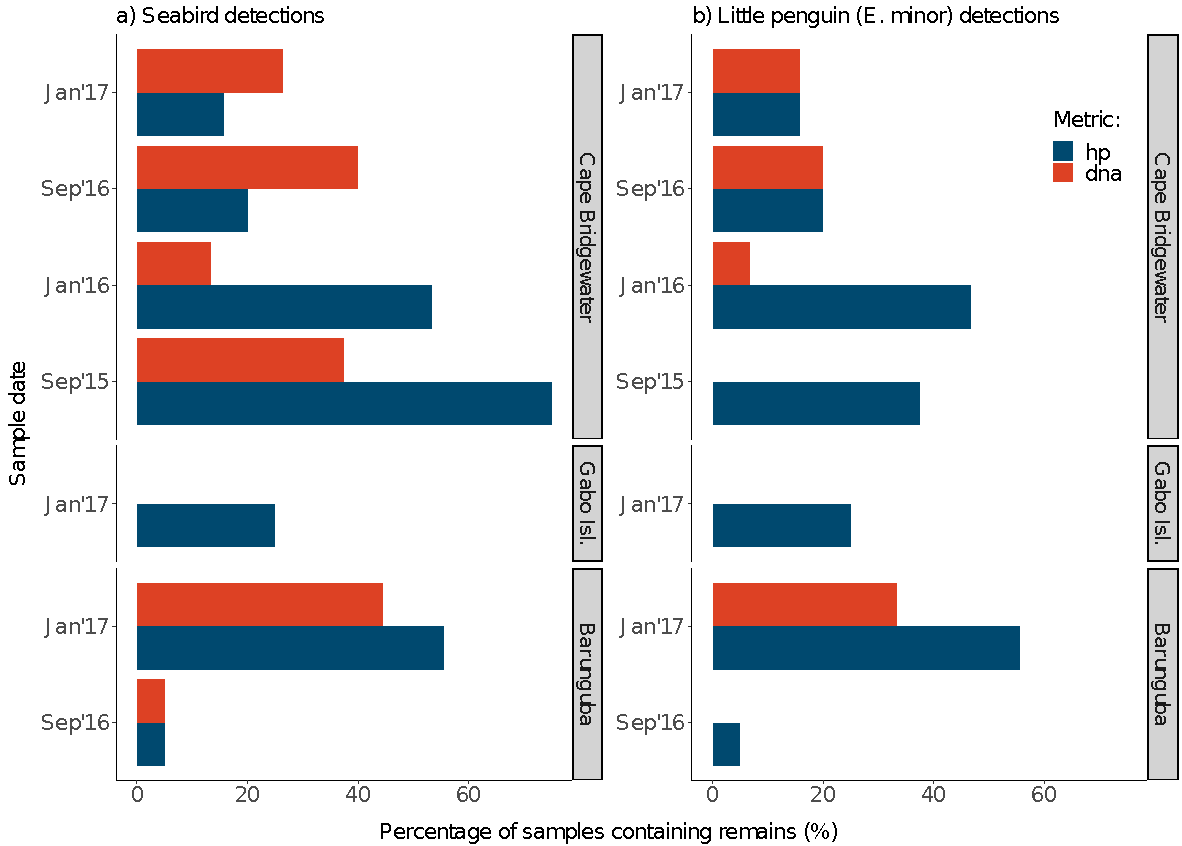
|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Sample ID** | **Total** | **Sequence 1** | **Sequence 2** | **Sequence 3** |
| CBW1\_16\_7\_S78\_Bird12sB | 5520 | 5315 |  |  |
| CBW1\_17\_10\_S93\_Bird12sB | 159 | 151 |  |  |
| CBW9\_16\_5\_S87\_Bird12sB | 6348 | 5210 | 454 | 400 |
| MI1\_17\_5\_S102\_Bird12sB | 4415 | 4289 |  |  |
| MI1\_17\_6\_S103\_Bird12sB | 894 | 589 | 262 |  |
| MI1\_17\_8\_S104\_Bird12sB | 206 | 200 |  |  |

**Table S6.** Generalised linear model with binomial distribution for seabird and little penguin detections in long-nosed fur seal scats across sampling groups (combined location and sampling time; Fig. S2 & S3) and methods – seabird diagnostic morphological remains or genetic material (significant variables in **bold**,P > 0.05).

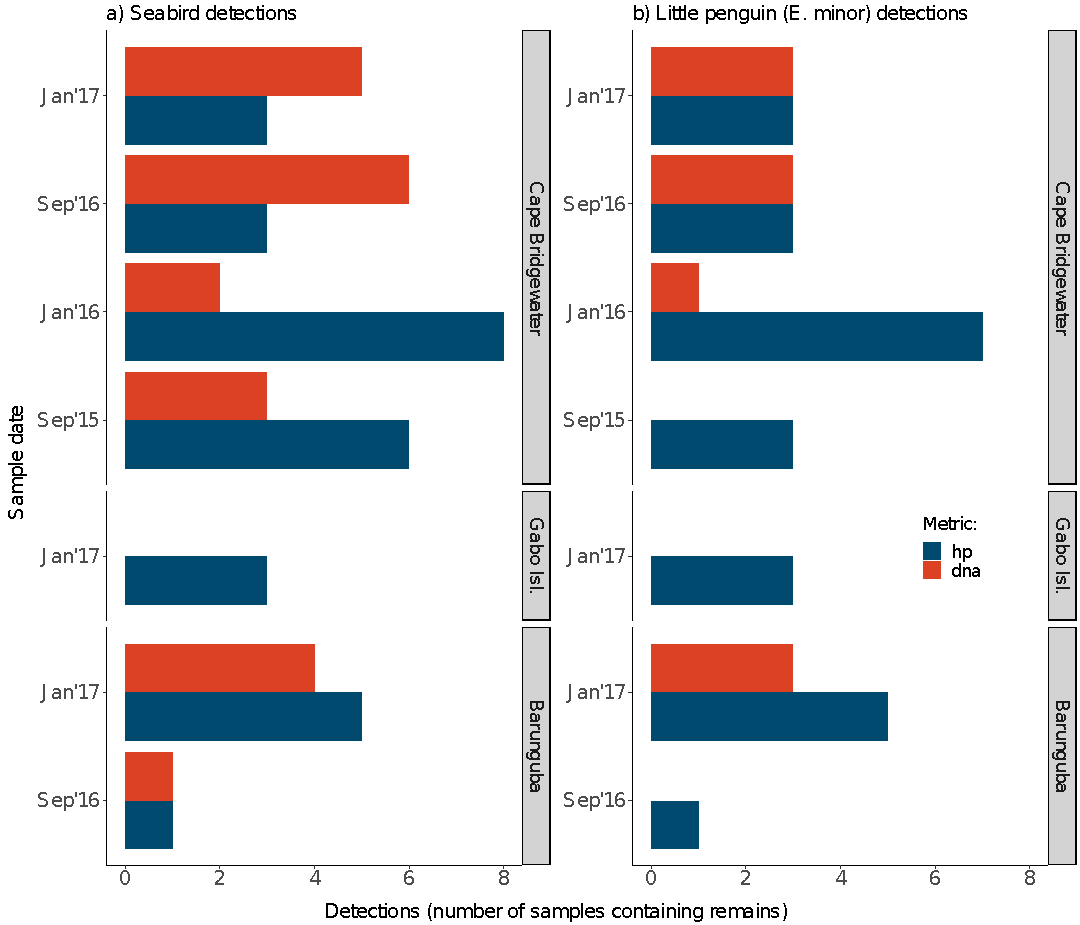
|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| **Model** | **Data type** | **Explanatory variables** | **df** | **Residual deviance** | **Residual df** | **Deviance** | **P** |
| Seabird | Detection (yes/no) | Method | 1 | 0.2081 | 98 | 114.403 | 0.6483 |
|  | *binomial* | 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 |

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



**Figure S2.** 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 S3.** Numerical detections of seabirds by method (hp = hard-part analysis, dna = DNA metabarcoding), location, and sample date (month’ year), for a) all seabirds and b) little penguins (*E. minor*).

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