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# Quantifying wildlife conflicts with metabarcoding and traditional dietary analyses

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| Abstract: | Wildlife conflicts require robust quantitative data on incidence and impacts, particularly among species of conservation and cultural concern. We present a multi-assay framework to quantify predation across systems and wildlife conflict scenarios, applied in southeastern Australian scenario where complex management implications and calls for predator culling are growing despite a paucity of predation data. We apply two ecological surveillance techniques to predator diets – traditional morphometric (hard-part) and DNA metabarcoding (genetic) analyses – to provide managers with estimated predation incidence, number of species impacted and prey relative importance to the predator. We explore haplotype diversity of prey DNA obtained for a species of conservation concern as a preliminary estimate of individuals consumed. We estimate the incidence of predation on seabirds by recovering and protected long-nosed fur seals (Arctocephalus forsteri) ranges from 9–29% of samples and included up to 6 prey species. The most common seabird prey – the culturally valued little penguin (Eudyptula minor) occurred in 6–25% of samples. This is higher than previously reported from traditional morphological assays alone. DNA metabarcoding proved more sensitive in identifying additional seabird taxa and provided relative quantitative information where multiple prey species occur within a sample. Polymorphism analysis of consumed little penguin DNA identified five distinct mitochondrial haplotypes – representing a minimum of 16 individual penguins consumed across 10 fur seal scat samples. We recommend rapid uptake and development of cost-effective genetic techniques and broader spatiotemporal sampling of fur seal diets to further quantify predation incidences and hotspots of concern for wildlife conflict management. |
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**Running head:** Quantifying wildlife conflicts


## Abstract

Wildlife conflicts require robust quantitative data on incidence and impacts, particularly among species of conservation and cultural concern. We present a multi-assay framework to quantify predation across systems and wildlife conflict scenarios, applied in southeastern Australian scenario where complex management implications and calls for predator culling are growing despite a paucity of predation data. We apply two ecological surveillance techniques to predator diets – traditional morphometric (hard-part) and DNA metabarcoding (genetic) analyses – to provide managers with estimated predation incidence, number of species impacted and prey relative importance to the predator. We explore haplotype diversity of prey DNA obtained for a species of conservation concern as a preliminary estimate of individuals consumed. We estimate the incidence of predation on seabirds by recovering and protected long-nosed fur seals (*Arctocephalus forsteri*) ranges from 9–29% of samples and included up to 6 prey species. The most common seabird prey – the culturally

19 valued little penguin (*Eudyptula minor*) occurred in 6–25% of samples. This is higher than
20 previously reported from traditional morphological assays alone. DNA metabarcoding proved
21 more sensitive in identifying additional seabird taxa and provided relative quantitative
22 information where multiple prey species occur within a sample. Polymorphism analysis of
23 consumed little penguin DNA identified five distinct mitochondrial haplotypes – representing
24 a minimum of 16 individual penguins consumed across 10 fur seal scat samples. We
25 recommend rapid uptake and development of cost-effective genetic techniques and broader
26 spatiotemporal sampling of fur seal diets to further quantify predation incidences and
27 hotspots of concern for wildlife conflict management.
28

29 **Introduction**

30

31 New wildlife conservation and management scenarios are emerging and increasing during the
32 Anthropocene as some species experience population increases through successful
33 conservation efforts, while others continue to decline (Roman et al., 2015; Marshall et al.,
34 2016; Cammen et al., 2019). Complex management scenarios arise when a species recovery
35 results in negative interactions with other species of value, whether that reflects a trophic role
36 in the ecosystem, conservation status, community connection or economic opportunity

(Marshall et al., 2016) – for example between killer whales, sea otters and salmon (Estes et al., 1998; Williams et al., 2011); New Zealand sea lions and yellow-eyed penguins (Lalas et al., 2007); wolves and caribou (Hervieux et al., 2014). The interactions are natural but present a need for accurate information on natural predation levels and impacts to prey (Hervieux et al., 2014) to avoid unjustified persecution of the predator (Granquist et al., 2018), and for effective management of all species concerned (Marshall et al., 2016).

A key goal of investigating predator prey interactions involves determining prey inter- and intra-specific diversity, dietary proportions, and abundances or biomass consumed by the predator (reviewed by Pompanon et al., 2012). Developments in eDNA extraction and metabarcoding techniques are demonstrating reliability for this level of environmental monitoring (Thomsen & Willerslev 2015) by: (i) identifying prey at high taxonomic resolution and when missed by other methods (Bowen & Iverson, 2013; Stat et al. 2019); (ii) estimating dietary proportions, reconstructing biomass and abundances of prey consumed through relative genetic importance (Deagle et al., 2019); (iii) identifying species' intraspecific genetic diversity within environmental samples for wildlife forensics and population estimation (Sigsgaard et al., 2016; Seersholm et al., 2018; Tsuji et al., 2020).

One wildlife conflict in southeastern Australia involves the recovery of long-nosed fur seals (*Arctocephalus forsteri*) and their potential to threaten populations of little penguin

(*Eudyptula minor*). The fur seals were decimated by fur trade through the 1800's and culling into the late 1900's due to perceived competition for resources with fishermen (Shaughnessy et al., 1999). Long-nosed fur seals are the only mainland Australian seal species with increasing population trends, reported at 97,200 in the state of South Australia (2013–14 census; Shaughnessy et al., 2015) where an estimated 83% of their recorded pup production occurs. While the original population size is unknown, harvesting records suggest that the current population represents a small fraction of that prior to European colonisation and exploitation (Ling, 2014).

Little penguins are a popular tourist attraction and valued species to communities across southern Australia (Tisdell & Wilson, 2012), and estimated at 470,000 individuals (BirdLife International, 2021). Yet, 60% of sites have unknown population trends, 29% of colonies are declining, most persist on offshore islands in southern Australia and are difficult to census (BirdLife International, 2021). Major contributors to decline include: (i) changes in land-use and predators introduced by European settlers (Dann, 1991; Kirkwood et al., 2014), (ii) susceptibility to hyperthermia during more frequent terrestrial heat waves (Lauren Tworkowski, La Trobe University, unpublished data), and (iii) large-scale changes to food webs caused by climate change and competition with fisheries (Ropert-Coudert et al., 2019). Little penguins and other seabirds were identified in juvenile, sub-adult, and adult male long-

nosed fur seals diets, at two locations in southern Australia and at relatively low frequencies (Page et al. 2005; Hardy et al. 2017; Goldsworthy et al. 2019). However, the number of penguins consumed, and the impact are unknown, particularly for 'unquantifiable remains', such as feathers and when prey are not consumed whole. Page et al. (2005) proposed a single scat containing feathers was equivalent to a single bird consumed; however, mass-balanced trophodynamic modelling in the Great Australian Bight marine ecosystem suggested that this likely overestimated predation (Goldsworthy et al. 2013).

Both species are federally protected and garner significant cultural and conservation value (*Environment Protection and Biodiversity Conservation Act*, 1975 & 1999), albeit listed as 'Least Concern' by the IUCN Redlist (IUCN 2020). The recovery of Australian seal species continues to conflict with some communities and fisheries (Shaughnessy et al., 2003; Goldsworthy & Page, 2007; Cummings et al., 2019) due to their potential predation impacts to prey species. While it is realistic to expect high levels of predation to affect prey population size or behaviour (Visser et al., 2008), persistent campaigning to cull the long-nosed fur seal population in South Australia are growing despite an absence of quantitative information (Goldsworthy et al. 2019).

To assess predation incidence and potential impact on little penguin and seabirds across southeastern Australia, we apply two surveillance techniques – morphometric (hard-part) and DNA metabarcoding (genetic) assays – to long-nosed fur seal scats. Due to known

biological and methodological differences in dietary information (Casper et al., 2007; Tollit et al., 2009), and differences in the quantities of DNA obtained from hard-parts compared to soft tissues (McDonald & Griffith, 2011), we consider these techniques complementary. Specifically, (i) we compare overall seabird and little penguin detection rates across groups of samples, rather than sample-by-sample; (ii) we investigate the diversity and relative importance of seabirds consumed by long-nosed fur seals; and (iii) we explore a minimum estimate of penguin abundance consumed by analyzing mitochondrial haplotype diversity among little penguin DNA obtained.

## Methods

### *Sample collection*

Individual predator scat samples (n = 99) were collected across multiple time points from four long-nosed fur seal breeding colonies in Bass Strait and NSW, southeastern Australia (Fig. 1 & 2). Pup abundances are illustrated as a conventional proxy for relative seal population (Fig. 1; Appendix S1.1). Most samples were collected from the two larger colonies, Barunguba and Cape Bridgewater, in spring (September) 2016 and summer

(January) 2017, with additional samples included from spring 2015 and summer 2016 at Cape Bridgewater. Samples from Gabo Island were collected from summer 2017. One sample was opportunistically collected from Deen Maar Island and included in assays. Sample sizes resulted from balancing adequate replication per site with availability of fresh samples.

Whole and fresh (< 48 h old) scats were sampled to minimise bias from differential DNA degradation or partial loss of material. Whole scats were thoroughly mixed with individual disposable spatulas at point of collection and a 2 mL subsample was taken from each field-homogenized scat for DNA-based analyses of prey remains. The remaining whole scats were collected for analyses of morphological prey remains, using individual, zip-lock bags. Samples were stored within hours of collection between -10˚ and -20˚C in portable freezers (WAECO) for up to 7 d in the field and transferred to -20˚C freezer facilities.

*Identification of seabird morphological and genetic remains*

All prey items were identified from hard-parts using methods described by Page et al. (2005). Birds were identified using feathers and other remains such as feet, flippers, and heads (Fig. 2, Appendix S1.2).

Prey DNA extractions used 250 mg of faecal subsamples and MoBio PowerSoil® DNA Isolation Kits (www.mobio.com) with modifications to the manufacturer's instructions made in response to extraction optimisation (Appendix S1.3). DNA was eluted in 10 mM Tris buffer, MoBio PowerSoil® C6 solution, (www.mobio.com) and stored at -20˚C. Nuclear DNA for positive controls was extracted from muscle tissue (25 mg) of a domestic chicken

133 (*Gallus gallus domesticus*) and a little penguin using with Bioline Isolate II Genomic DNA
134 Kits (www.bioline.com/us/) as per manufacturer instructions.
135 In total, 99 faecal DNA sample extracts at two DNA concentrations (neat and 1:10
136 dilutions), as well as extraction blanks (n = 5), PCR blanks (n = 4), and positive controls (n =
137 2) were screened in duplicates by diagnostic endpoint PCR (dPCR) using the Bird12sa/h
138 assay (Cooper, 1994) (Table S1 & S2, Appendix S1). Duplicate dPCR products were run on
139 1.5% agarose gels to determine the presence/absence of amplified target bird DNA in each
140 duplicate. A total of 32 samples showed target amplicons in both or one duplicate, but not in
141 the extraction and PCR controls. We sequenced each of the 32 samples that tested positive
142 for birds, and two extraction blanks and one positive control (n = 35 samples for sequencing).
143 A single-step fusion tagging PCR procedure was used to attach and assign unique MID
144 (Multiplex IDentifier) tag combinations, next generation sequencing (NGS) adaptors and the
145 Bird12sa/h assay. The sequencing workflow – single-step fusion PCR (Appendix S1), library
146 build, sequencing (150 bp paired-end Illumina Miseq: v2 Nano 150 bp) and demultiplexing –
147 was performed by Ramaciotti Centre for Genomics, University of New South Wales.
148 Bioinformatics and sequence quality filtering procedures are described in
149 reproducible detail in Appendix S1.3. We used Geneious R8.1.5 (Kearse et al., 2012) to
150 merge the paired-end sequences (2x ~150 bp fragments, with overlap of 70 bp) and retain
151 only those with exact flanking sequences – MID tags, primers, and adapters. Primers,

adapters, and tags were removed to leave the complete target sequences in each sample. These were quality filtered and clustered into molecular operational taxonomic units (OTUs) using *UPARSE* algorithm and performed in *USEARCH* (Edgar, 2010; Edgar & Flyvbjerg, 2015). Low abundance sequences (below a threshold of 1% of the total abundance of all unique sequences) were removed to reduce the occurrence of sequencing error and chimeras, and sequences were then clustered using a 97% similarity criterion (similar to Berry et al., 2017). Thus 7370 unique seabird DNA sequences, representing a total of 64,700 disaggregated bird sequences, were parsed to standard sequence filtering and OTU clustering pipeline (with cluster size threshold value of 73), resulting in 47,478 filtered sequences across 99 samples, and these were clustered to 5 OTUs.

Consensus sequences for each OTU were queried against the National Center for Biotechnology Information's (NCBI) GenBank nucleotide database using the algorithm BLASTn (Basic Local Alignment Search Tool) (Benson et al., 2005). The resulting 'blasted' sequences were assigned to taxa, following criteria and taxonomic reference databases outlined in Hardy et al. (2017) and Appendix S1.3 (Table S3). These criteria maximised confidence in making a taxonomic identification and minimised risk of false positives.

169 *Haplotype polymorphism analysis*

170

171 We sought to identify a minimum number of individual little penguin by exploring mtDNA
172 haplotypes from 12S rRNA sequences obtained. While dependent on sequence fidelity, such
173 approaches have been used to explore intraspecific diversity (similar to Seersholm et al.,
174 2018). We imported the quality-filtered file containing 47,478 seabird DNA sequences,
175 produced just prior to OTU clustering, we disaggregated and matched these sequences in
176 relation to sample identifier and formed these into clusters of unique sequences in Geneious.
177 From the six samples that contained abundant penguin DNA (Table S4), we selected the nine
178 most abundant unique sequences. Each of these represents a sequence abundance of greater
179 than 7.5% of the total sequence abundance of the sample (Table S5). This process was used
180 to exclude beyond reasonable doubt, any further sequences that could be attributed to
181 sequencing error.

182 We produced a minimum spanning haplotype network using the software *PopART*
183 (Leigh & Bryant, 2015) from an alignment of these nine penguin sequences to visualise
184 relationships between haplotypes consumed, and between samples from the different
185 locations sampled. All samples containing little penguin DNA (n = 10) were then searched

for the presence of identified haplotypes. We estimated the number of penguins likely consumed based on the number of haplotypes within each sample. Additionally, as samples were collected across multiple days from each location and sampling time, we treated each sample as from distinct predators or predation events.

*Statistical analyses*

To account for different sampling times and locations, samples were assigned one of seven unique grouping factors that combined location and time (e.g., Barunguba, January 2017). The single scat sample collected from Deen Maar Island was not included in statistical tests, but results were reported for future comparisons. The overall detection rates of seabirds and specifically little penguins using different dietary analysis techniques (hard-part vs. DNA; Table S6, Appendix S2) were examined using two generalised linear models (GLMs), constructed in the *stats* package in R version 4.0.3 (R Core Team, 2020). A binomial error distribution for presence-absence data was used and additive term included to account for long-nosed fur seal group (location and time). Model fit was assessed using deviance explained and variable significance ($p < 0.05$).

## Results

Overall, the detection rates of seabirds were statistically similar for each method (Fig. 3, Table S3). However, DNA metabarcoding offered additional information: (i) absolute and relative abundance information for amounts of DNA recovered (Fig. S1 & S2, Table S4), (ii) improved sensitivity in detecting multiple prey taxa within a single scat (Fig. 4), and (iii) exploration of little penguin 12S rRNA genetic diversity enabled the estimation a minimum number of predated penguins to the be estimated (Fig. 5, Table S5).

### *Comparing diagnostic hard-part and genetic assays*

Seabirds were detected in 29.3% (n = 29) of samples using diagnostic hard-parts, and 21.2% (n = 21) of samples using DNA metabarcoding (Fig. 3a). Most of these detections were little penguins, detected in 25.3% (n = 25) of samples with hard-parts and 10.1% (n = 10) of samples with DNA (Fig. 3b). Of these positive detections obtained after quality control and filtering, the majority of DNA sequences for all seabird taxa were identified in 9 out of 21

220 samples, and for little penguins in 6 out of 10 samples, relative to the total DNA obtained for
221 each taxon (Figs 3 & S1, Table S4), and providing a lower conservative estimate for
222 predation incidence. The same seabird taxa were detected by simultaneously in half the
223 positive samples (n = 10), and half of these (n = 5) contained both little penguin hard-parts
224 and DNA (Fig. 3). Five samples contained DNA and hard-parts that did not belong to the
225 same seabird taxon (Fig. 4), and the remaining positive samples from each method represent
226 a detection made by one method alone (n = 6 for DNA, and n = 13 for hard-parts). The
227 combined proportion of samples that were positive for seabirds was 40% (n = 40), and 30%
228 for little penguins (n = 30).

229 Mean detection rates were statistically similar for both methods for seabirds and
230 penguins (Table S6). There was greater variability in detection rates across locations using
231 hard-parts compared to DNA (Fig. S3). Minor, albeit statistically significant, differences
232 were observed across sampling groups for seabird detection rates (Fig. S3a, Table S3), but
233 not for penguins (Fig. S3b) (GLM binomial seabird detection ~ location: p-value = 0.017;
234 Table S3). Detection variability was high across sampling locations, times and methods used
235 (Fig. S3a). Little penguins were detected by hard-parts from Gabo Island samples, however

236 only a few sequences of little penguin DNA were detected at Gabo Island and Deen Maar
237 Island, and these sequences did not pass DNA quality filtering.
238
239 *Seabird diversity in long-nosed fur seal diets*
240
241 DNA-based metabarcoding was more sensitive in detecting taxonomic mixtures in scat
242 samples compared to hard-part analysis (Fig. 4), with 2 distinct seabird taxa detected in 5
243 samples and a single seabird taxon in the remaining 16 samples. No samples contained more
244 than one identified bird taxon using diagnostic hard-parts (Fig. 4a). Little penguins were the
245 main seabird prey species detected using both analyses (Fig. 4), and this was reflected in all
246 data from DNA – frequency of occurrence (Fig. 4), total abundance of sequences (Fig. S1),
247 and relative sequence abundance (Fig. S2).
248 Morphological analysis revealed two additional taxa: a shearwater family group
249 (Procellaridae spp.) (n = 2 samples), and Australasian gannet (*Morus serrator*) (n = 1) (Fig.
250 4). DNA metabarcoding detected abundant DNA from two distinct shearwater taxa, also at
251 family-level, taxa in 5% (n = 5) and 9.1% (n = 9) of samples, respectively (Tables S3 & S4,
252 Appendix S2). Black-browed albatross (*Thalassarche melanophris*) and greater crested tern

253 (*Sterna bergii*) occurred in one sample apiece (Fig. 4, Tables S3 & S4). Parallel use of both
254 DNA metabarcoding and hard-part analysis revealed a greater diversity of taxa than either
255 method alone.
256
257 *Towards quantifying little penguin consumption*
258
259 From the six scats containing abundant penguin DNA, a total of five mtDNA haplotypes
260 were identified in samples from Cape Bridgewater (haplotypes 1–2 and 4–5) and Barunguba
261 (haplotypes 1–3) (Fig. 5a). Only haplotype 3 was unique to Barunguba, the remaining five
262 haplotypes were detected at both sites (Figs. 5b). Haplotype 1 was identified three different
263 single sources of DNA from penguins from southeastern Australia (see reference material in
264 Appendix S1.4). Across all ten penguin positive scat samples, six contained a single
265 haplotype, whilst the remaining four contained between 2–4 haplotypes (Fig. 5b). Taking two
266 distinct genetic haplotypes present within a sample to represent at least two distinct
267 individual birds consumed, we estimate at least 16 individual penguins were consumed across
268 the 99 scat samples, from two sampling locations and multiple seasons.
269

## Discussion

Validating and applying modern surveillance tools, such as metabarcoding, to complex conservation scenarios can better inform decision making, also providing transferable . We leveraged metabarcoding alongside traditional diet analysis methods, to investigate a wildlife conflict in southeastern Australia. We provide an updated and improved predation prevalence range for seabirds overall (9–29%) and little penguins (6–25%) in long-nosed fur seal diet. We confirm that little penguins remain the most frequently consumed seabird by long-nosed fur seals in comparison to other avian taxa. Here, DNA metabarcoding also offered key advantages over morphological analysis – quantitative information on: (i) absolute and relative abundances of taxa recovered using DNA, (ii) detection of multiple prey taxa within a single scat sample, and (iii) estimates of at least 16 penguins by exploring the spatial and temporal distribution of haplotypes.

Previous studies using either assay techniques have identified little penguin remains at relatively low frequencies in relation to seals' total diets (5.9% in Page et al. 2005, <2% in Hardy et al. 2017, ~13% in Goldsworthy et al. 2019). The lower range of estimates observed here (9% of samples for seabirds, 6% for penguins) and based on samples containing large

quantities of prey DNA corroborate these previously reported predation rates for the region. However, the upper range of estimates observed in this study (10% and 25% of samples, respectively for DNA and hard-parts) and recent crashes in little penguin colonies (R. R. McIntosh, pers. comm.) signal a need for broader and increased monitoring of predation mortality. Predatory behaviours could be transmitted to other predator populations, particularly in response to food web disruption under ocean warming and changes in prey availability and this could have cascading effects on penguin populations. Analysis of the predator's total diet is also warranted to gauge the relative importance of different prey items, besides seabirds.

While both metabarcoding and morphological methods provided statistically similar results, we emphasize that these represent complementary but quasi-independent assays of predation. Different detection rates reported between these methods in this study are also common across a broad range of predator and prey taxa (Tollit et al., 2009; Zarzoso-Lacoste et al., 2013). Many methodological and biological factors differentially affect the recovery of diagnostic genetic and morphological materials. In another pinniped species, prey soft tissues had a gut passage rate of 48 hours and up to 7 days for hard-parts (Tollit et al., 2009). Soft tissues may contain greater DNA quantities and concentrations, particularly mitochondrial

304 (Mumma et al., 2016; Granquist et al., 2018), and could be preferentially amplified compared
305 to DNA available from hard-parts, (i.e., feather, fur, or bone). DNA from these tissues is of
306 poor quality and requires a different process for extraction (McDonald & Griffith, 2011;
307 Rothe & Nagy, 2016). Typically, studies report higher detection rates from genetic assays
308 compared to morphological analyses (Egeter et al., 2015; Mumma et al., 2016), but the lower
309 detection rates here are consistent with other pinniped studies (Tollit et al., 2009) and with
310 over-representation of feathers across fur seal scats (Goldsworthy et al., 2019), explaining the
311 higher detection rate from these hard-parts.

312 Distinct variation in penguin metabarcodes obtained, beyond that attributed to
313 sequencing error, led us to explore how many individuals might be within the data (similar to
314 Seersholm et al., 2018 and Tsuji et al., 2020). While not the ideal locus to explore intra-
315 species diversity, we nonetheless identified 5 haplotypes consumed, across time and space
316 this suggested at least 16 penguins were consumed. This is very likely an underestimate of
317 predation, firstly because we used the highly conserved gene for 12S ribosomal RNA (Banks
318 et al., 2002), selected for proven reliability in detecting seabirds (Hardy et al. 2017).
319 Targeting alternative markers (variable barcodes or microsatellites) could reveal greater
320 genetic diversity, however we did not succeed with a COI barcode (Appendix S1.2). To avoid
321 interpreting error (PCR or sequencing) as separate individuals we followed stringent
322 sequence quality and abundance filtering to exclude the possibility of false positives beyond

sequencing error, and we likely excluded several low DNA-abundance, true positives (Deagle et al., 2013).

Most scats with penguin DNA contained a single haplotype, however four scats contained up to 4 distinct genetic haplotypes, suggesting that long-nosed fur seals can consume multiple penguins in a single foraging trip or within 48h of sampling. This result provides a significantly more reliable method of estimating predation incidence and impact than previous assumptions that each scat containing feathers corresponded to a single bird (Page et al., 2005, Mumma et al. 2016). If little penguin predation becomes an important individual foraging strategy even for some long-nosed fur seals, this could have serious negative impacts for isolated penguin populations. DNA-based methods are transferable across systems and offer vast potential for technological and methodological improvements (Tsuji et al., 2020) over traditional morphological diet assays.

Finally, our results demonstrate a need for research and development of techniques at the nexus of population genetics and environmental sampling – including screening for predator genetic diversity to identify individuals in a population contributing to predation of a sensitive or valuable species (Wegge et al., 2012), developing species-specific probes using older and cheaper technology (Fox et al., 2012), cross-validation of eDNA data with quantitative PCR (Murray et al. 2011), or development of penguin-specific DNA-to-tissue-based correction factors could provide consumed biomass information (Thomas et al., 2014).

342 Additionally, predator impacts need to be considered and managed within up-to-date
343 cumulative impact assessments for threats. We have delivered an important step towards this
344 for little penguins in south-eastern Australia. Similar to other wildlife conflict situations,
345 endemic predation is natural and often habitat degradation, environmental change and
346 invasive species are more significant sources of impact to susceptible species (Hervieux et
347 al., 2014; Marshall et al., 2016; Ropert-Coudert et al., 2019).
348

349 **Supporting Information**

350

351 PINP_Bird_supplement.docx.

352

353 **Data Availability**

354

355 Datasets and code used for figures and analyses will be placed in public online repository
356 upon acceptance of this manuscript for publication.

357

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553

554 **Figure Legends**

555

556 **Figure 1.** a) Long-nosed fur seal scat collection sites (n = total number of samples), including
557 pup abundances for sampling locations (McIntosh et al., 2014) as an index of seal population
558 relative importance of sites. Sampled sites were: Cape Bridgewater (38.3013˚ S, 141.4062˚
559 E) and nearby Deen Maar Island (formerly Lady Julia Percy Island, 38.4161˚ S, 142.0038˚ E)
560 from western Bass Strait, Victoria; Gabo Island in eastern Bass Strait, Victoria (37.5649˚ S,
561 149.9133˚ E); and Barunguba (formerly known as Montague Island 36.2510˚ S, 150.2270˚ E)
562 at the northeastern breeding range in New South Wales (NSW). Species distributions shown
563 for b) long-nosed fur seals and c) little penguins (data from ALA, 2019).

564

565 **Figure 2.** a) A long-nosed fur seal, *Arctocephalus forsteri*, from Barunguba, NSW; b) the
566 little penguin, *Eudyptula minor*, often burrowing near fur seal colonies; c) and d) seabird
567 remains are conspicuous at long-nosed fur seal haul-outs and colonies, among scats and
568 regurgitates.

569

570 **Figure 3.** Detections of a) seabird and b) little penguin diagnostic hard-parts ('hp') and DNA
571 ('dna'), as a percentage of all long-nosed fur seal samples (n = 99). We report all genetic
572 sequences obtained from standard sequence quality control and filtering 'DNA (all)', and for
573 samples that contained large sequence quantities of sequences 'DNA (abundant)' (> 90% of
574 filtered sequences); and the number of samples that contained both the morphological and
575 genetic remains of the same seabird ('same taxon').

576

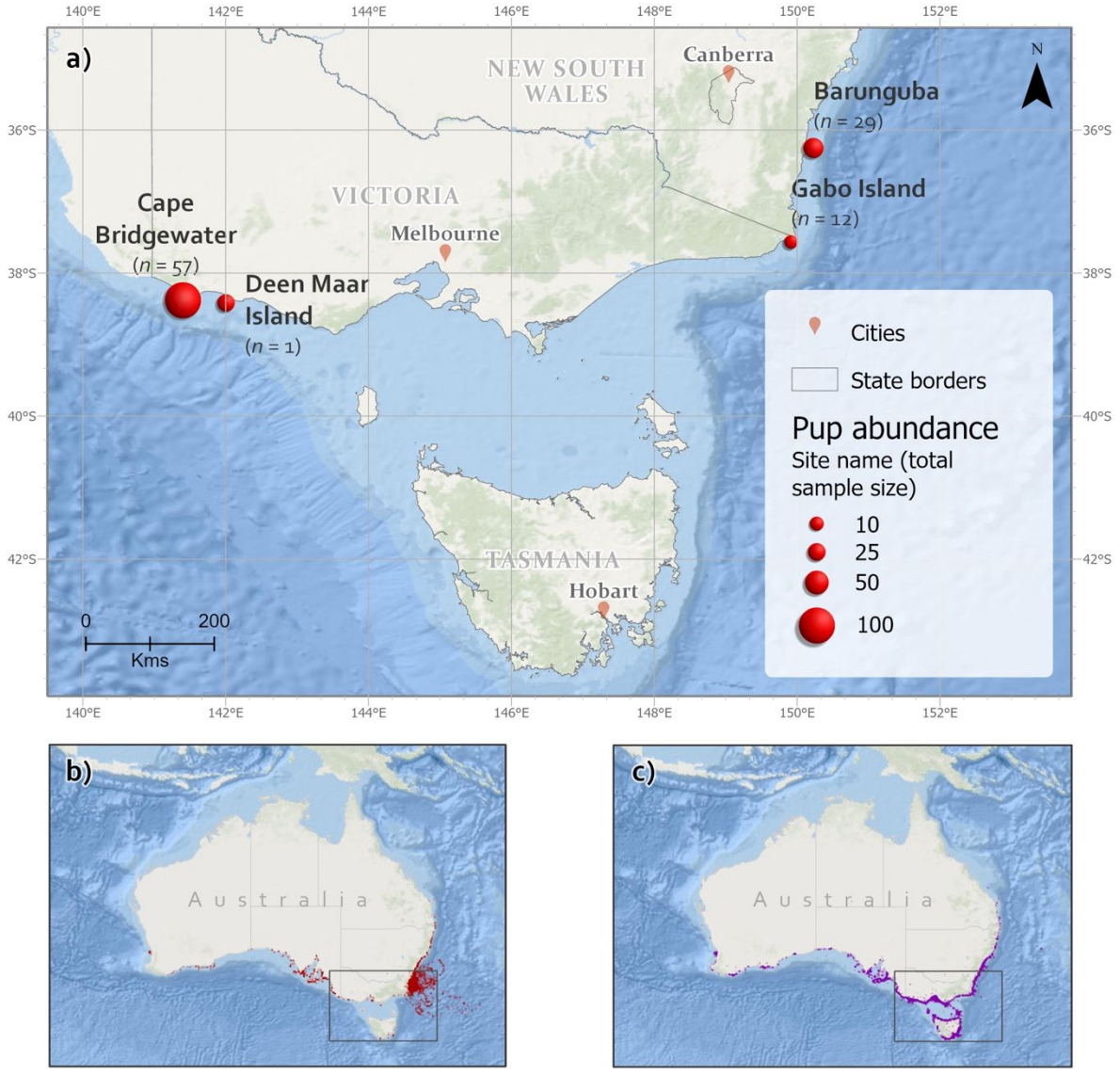
577 **Figure 4.** The diversity of seabirds identified in long-nosed fur seal samples: a) using hard-
578 part analyses (n = 29) and b) using DNA-based methods (n = 21). GI = Gabo Island.

579

580 **Figure 5.** Little penguin genetic diversity (for ~230 bp 12S rRNA gene) a) presented as a
581 minimum spanning network of five distinct haplotypes, and b) number of haplotypes
582 contained within each penguin-positive sample, including haplotype sequence abundances
583 within samples.

584 **Figures**

585




b) Long-nosed Fur Seal and c) Little Penguin distribution data

586

587 **Figure 1.**

588


589

590 **Figure 2.**

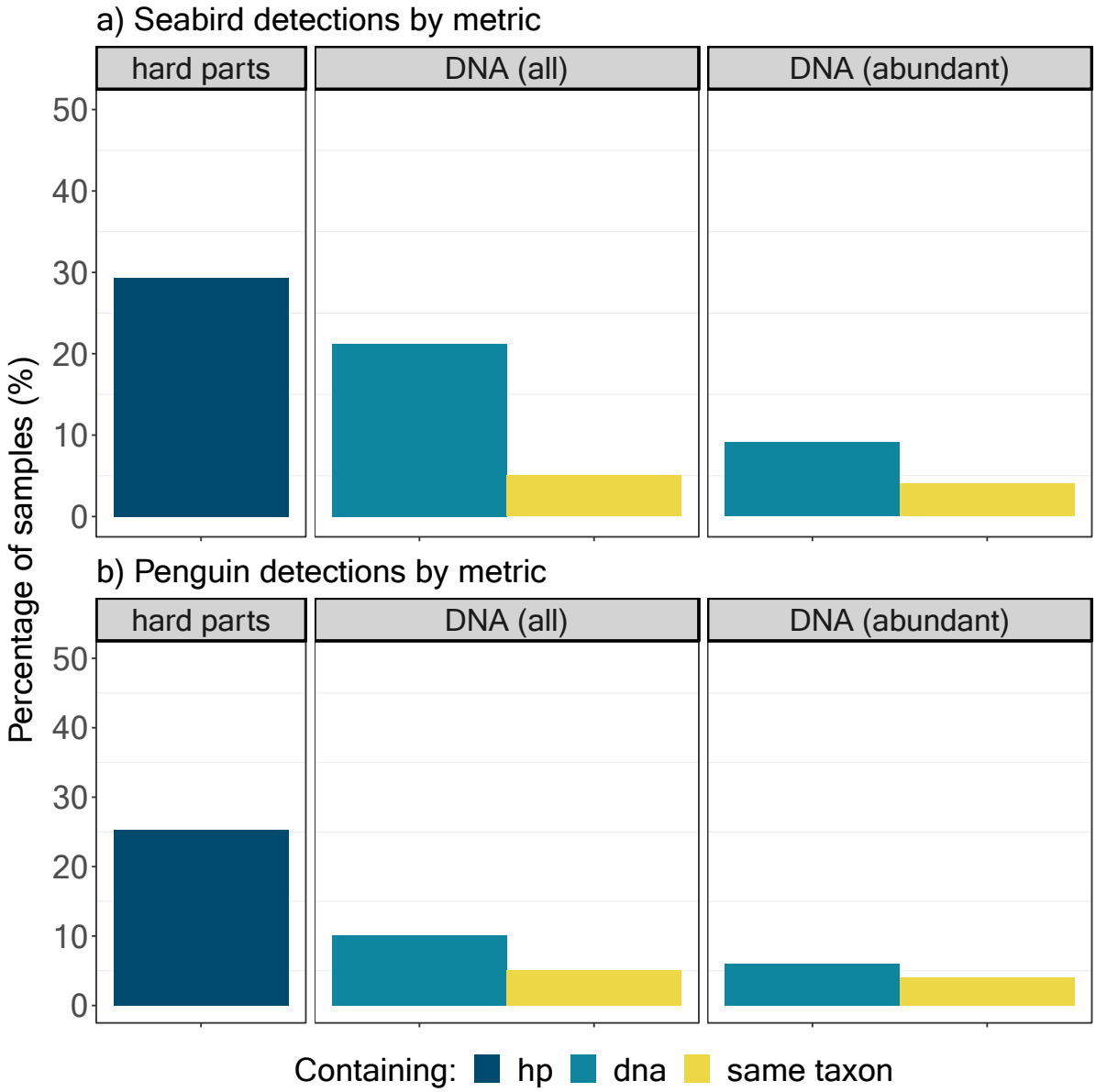

a) Seabird detections by metric
hard parts
DNA (all)
DNA (abundant)
50
40
30
20
10
0
b) Penguin detections by metric
hard parts
DNA (all)
DNA (abundant)
50
40
30
20
10
0
Percentage of samples (%)
Containing: hp dna same taxon

Figure 3.

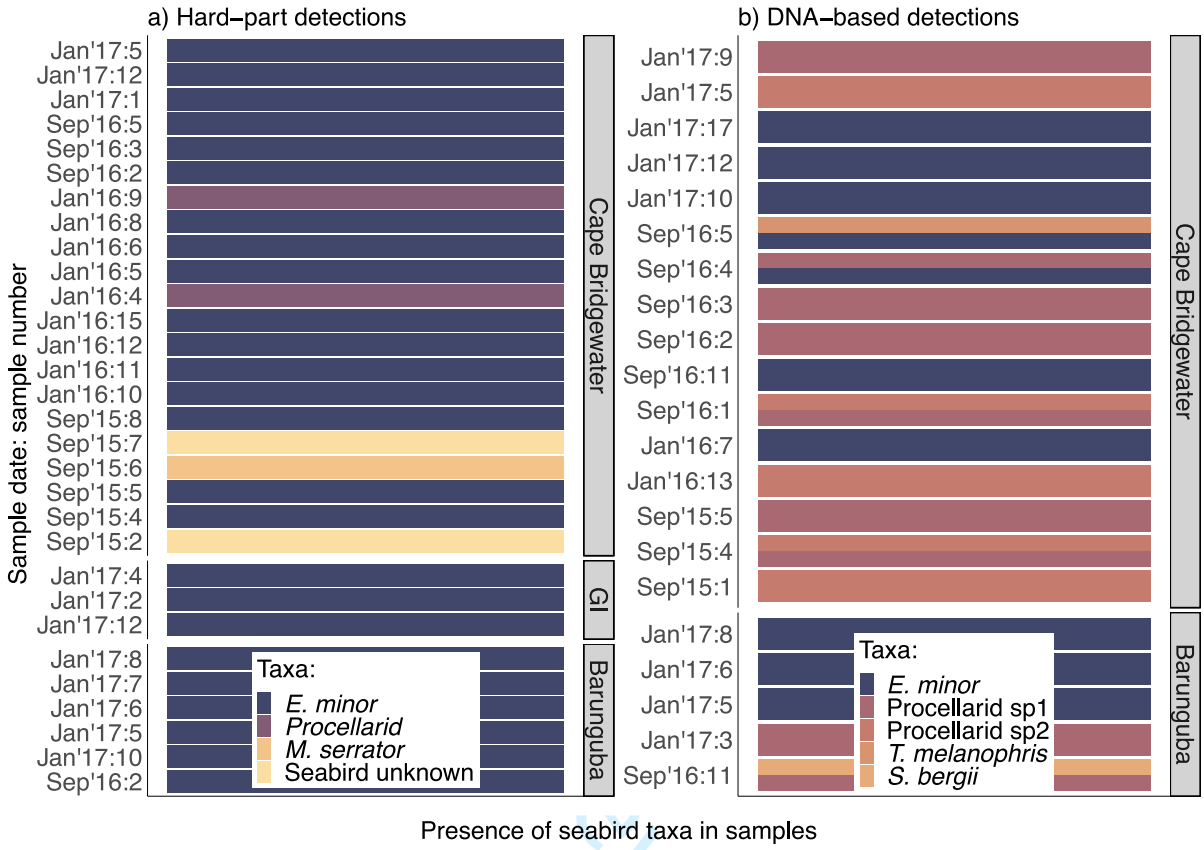



**Figure 4.**

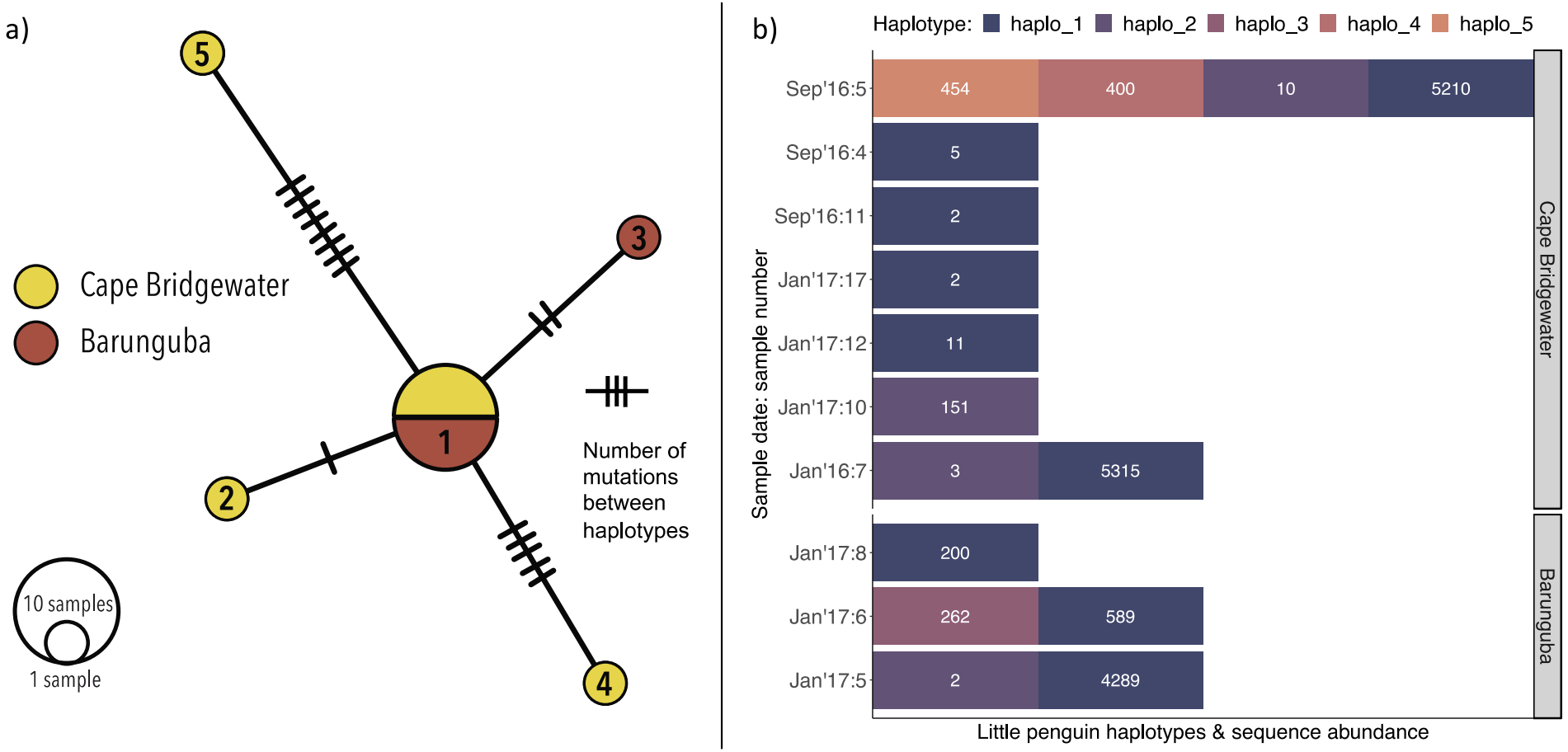

a)
5
3
Cape Bridgewater
Barunguba
1
2
Number of mutations between haplotypes
10 samples
1 sample
4
b)
Haplotype: haplo_1 haplo_2 haplo_3 haplo_4 haplo_5
Sep'16:5
Sep'16:4
Sep'16:11
Jan'17:17
Jan'17:12
Jan'17:10
Jan'16:7
Jan'17:8
Jan'17:6
Jan'17:5
454
400
10
5210
5
2
2
11
151
3
5315
200
262
589
2
4289
Sample date: sample number
Cape Bridgewater
Barunguba
Little penguin haplotypes & sequence abundance

**Figure 5.**

**Title:** Quantifying wildlife conflicts ~~by combining~~with ~~multi-assay~~ ~~eDNA~~ metabarcoding and traditional dietary analys~~ci~~is

**Running head:** Quantifying wildlife conflicts


## Abstract

Wildlife conflicts ~~interactions~~ require robust quantitative data on incidence and impacts, particularly among species of conservation and cultural concern. ~~Conflicts between iconic species are likely to increase with mounting pressures during the Anthropocene.~~ We ~~therefore~~ present a ~~modular~~ mu~~t~~lti-assay framework ~~for~~ to quantify~~ing~~ predation ~~broadly~~ across systems and wildlife conflict scenarios, applied in southeastern Australian scenario where complex management implications and calls for predator culling are growing despite a paucity of predation data. We ~~combine~~ apply two ecological surveillance techniques ~~applied~~ to predator diets ~~analysis,~~ – traditional morphometric (hard-part) and DNA metabarcoding (genetic) analyses~~,~~ – to provide managers with ~~an~~ estimated ~~incidence of~~ predation incidence, ~~the~~ number of species impacted and ~~quantitative information on~~ prey relative importance to the predator. ~~Further~~W~~, w~~e explore ~~perform a polymorphism analysis~~

onhaplotype diversity of obtained prey DNA obtained for a species of conservation concern toas a preliminary estimate of individuals the abundances consumed for a prey species of conservation concern. We apply this framework to an emerging wildlife conflict where complex management implications and calls for predator culling are growing in southeastern Australia, despite the paucity of predation data. We estimate that the incidence of predation on seabirds by recovering and protected long-nosed fur seals (*Arctocephalus forsteri*) of ranges from 9–29% of samples and across included up to 6 seabirdprey species,. The most common seabird prey – the culturally valued little penguin (*Eudyptula minor*) andoccurred in ranging from 6–25% of samplesfor their main seabird prey – the culturally valued little penguin (*Eudyptula minor*),. This is, and higher than previously reported from traditional morphological assays alone. DNA metabarcoding proved more sensitive in identifying additional seabird prey taxa and provided relative quantitative information where multiple prey species occur within a sample. Polymorphism analysis of consumed little penguin DNA identified five distinct mitochondrial haplotypes – representing a minimum of 21 16 individual penguins consumed across just 10 fur seal scat samples. We recommend rapid uptake and development of cost-effective genetic techniques and broader spatiotemporal sampling of predator fur seal diets to further quantify predation incidences and hotspots of

concern for wildlife conflict management ~~using the most cost-effective assaying techniques.~~

~~We~~ The ~~highlight the utility of DNA metabarcoding techniques~~ are highlighted, providing ~~in providing more reliable quantitative information on predation incidence and likely abundance of impacted species of conservation concern~~.

**Introduction** ~~930 words~~

~~Conflicts between iconic species are likely to increase with mounting human pressures on wildlife during the Anthropocene.~~ New wildlife conservation and ~~wildlife~~ management scenarios are emerging and increasing during the Anthropocene as some species experience population increases through successful conservation efforts, while others continue to decline ~~due to anthropogenic impacts~~ (Roman et al., 2015; Marshall et al., 2016; Cammen et al., 2019). Complex management scenarios arise when a species recovery results in negative interactions with other species of value, whether that ~~value~~ reflects a trophic role in the ecosystem, conservation status, community connection or economic ~~value~~ opportunity (Marshall et al., 2016) – for example between ~~. Prominent examples abound of conflicting predator-prey interactions among species of value:~~ killer whales, sea otters and salmon (Estes et al., 1998; Williams et al., 2011); New Zealand sea lions and yellow-eyed penguins (Lalas

**Commented [RM1]:** More reliable than what? I have deleted from sentence

**Commented [RM2]:** Reads repetitive with above (yellow), choose a better word if you like – resource?

et al., 2007); wolves and caribou (Hervieux et al., 2014). The interactions ~~themselves~~ are natural, ~~however they~~but present a need for accurate information on natural predation levels and impacts to prey (Hervieux et al., 2014) to avoid unjustified persecution of the predator (Granquist et al., 2018), and for effective management of all species concerned (Marshall et al., 2016).

~~The ultimate~~A key goal~~s in of~~ investigating ~~the incidence and impacts of~~ predator prey interactions involve~~s~~ determining prey inter- and intra-specific ~~identities~~diversity, dietary proportions, and abundances or biomass consumed by the predator (reviewed by Pompanon et al., 2012). Developments in eDNA extraction and metabarcoding techniques are demonstrating reliability for this level of environmental monitoring (Thomsen & Willerslev 2015~~; Stat et al. 2019~~) ~~by~~and utility for ~~achieving these goals by~~: (i) identifying ~~species~~ prey at high taxonomic resolution and when missed by other methods (Bowen & Iverson, 2013; Stat et al. 2019); (ii) estimating dietary proportions ~~and,~~ reconstructing biomass and abundances of prey consumed through relative genetic importance (~~Thomas et al., 2014;~~ Deagle et al., 2019~~; Cavallo et al., 2020~~); (iii) identifying species' intraspecific genetic diversity within environmental samples for wildlife forensic ~~purpose~~s and ~~sample~~ population estimation (Sigsgaard et al., 2016; Seersholm et al., 2018; Tsuji et al., 2020).

An emergingOne wildlife conflict in southeastern Australia involves the recovery of long-nosed fur sealseals (*Arctocephalus forsteri*) and their potential to threaten populations of the culturally valued little penguin (*Eudyptula minor*). The fur seals were decimated by massive over-exploitation during the fur trade through the 1800's for the fur trade and culling into the late 1900's due to perceived competition for resources with fishermen (Shaughnessy et al., 1999). Long-nosed fur seals are the only mainland Australian seal species with increasing population trends, reported at 97,200 in the state of South Australia (2013–14 census; Shaughnessy et al., 2015), and where an estimated 83% of their known recorded pup production occurs. Total estimates of mainland Australian seal populations prior to sealing were never made, however it is noteworthyWhile the original population size is unknown, harvesting records suggest that the the recent recovery ofcurrent long-nosed fur seals population likely represents a small fraction of their populationthat prior to European colonisation and exploitation (Ling, 2014).

Little penguins are a popular tourist attraction and locally valued species to communities across southern Australia (Tisdell & Wilson, 2012), with anand estimated at 470,000 little penguin individuals (BirdLife International, 2021). Yet, 60% of sites have unknown population trends, 29% of colonies are declineteriorating and, most persist on offshore islands in southern Australia where theyand are difficult to census (BirdLife International, 2021). Major threats and contributors to decline include: (i) changes in land-use

and land-based predators introduced by European settlers (Dann, 1991; Rout Kirkwood et al., 2014), (ii) inscreasing susceptibility to hyperthermia during increasingly more frequent terrestrial heat waves (Lauren Tworkowski, La Trobe University, unpublished data), and (iii) large-scale changes to foods webwebs caused by ocean warmingclimate change and competition with marine fisheries (Ropert-Coudert et al., 2019). Little penguins and other seabirds have beenwere identified in the diets of juveniles, sub-adult, and adult male long-nosed fur seals diets, at two locations in southern Australia and at relatively low frequencies (Page et al. 2005; Hardy et al. 2017; Goldsworthy et al. 2019). However, penguinthe number of penguins abundances consumed and predation the impacts have been difficult to estimate.estimatequantifyare unknown, particularly for . Page et al. (2005) estimated penguin abundance and biomass consumed based on the presence of distinguishing remains (1 skull and/or 1 pair of wings = 1 individual). For 'unquantifiable remains', such as feathers and when prey are not consumed whole.,. Page et al. (2005) proposed a single scat containing feathers was equivalent to a single bird consumed; h. However, mass-balanced trophodynamic modelling in the Great Australian Bight marine ecosystem suggested that previous estimates of penguin biomass consumed, and thus predator impacts were likely overestimatedthis resulted in likely overestimatesd of predation (Goldsworthy et al. 2013).

Both species are federally protected and garner significant cultural and conservation value (*Environment Protection and Biodiversity Conservation Act*, 1975 & 1999), albeit .

Both are also listed as 'Least Concern' by the IUCN Redlist (IUCN 2020). However, tThe recovery and protection of many Australian seal species continues to conflict with many some communities and fisheries (Shaughnessy et al., 2003; Goldsworthy & Page, 2007; Cummings et al., 2019) due to their potential predation impacts to prey species. While it is realistic to expect high levels of predation to affect prey population size or behaviour (Visser et al., 2008), conflict between long-nosed fur seals and little penguins has not been reliably quantified.is often perceived, . Yet, the perception of this conflict has ledadingd to persistent campaigning and vocal calls to cull the long-nosed fur seal population in South Australia are growing despite an absence of quantitative information (Goldsworthy et al. 2019). In the absence of quantitative information on interactions – the frequency and magnitude of impacts by long-nosed fur seals on little penguins are largely unknown.

WeWe To assess predation incidence and potential impact on little penguin and seabirds across southeastern Australia, we combine apply two ecological surveillance techniques – morphometric (hard-part) and DNA metabarcoding (genetic) assays – of to long-nosed fur seal scats – to assess predation incidence and potential impact on little penguin and seabirds across southeastern Australia. Due to known biological and methodological differences in dietary information (Casper et al., 2007; Tollit et al., 2009), and differences in the quantities of DNA obtained from hard-parts compared to soft tissues

(McDonald & Griffith, 2011), we consider ~~consider~~ these techniques ~~simultaneously~~ ~~informative~~complementary. ~~First~~to ~~these methods~~First, ~~w~~Specifically, (i) we ~~first aimed to~~ compare overall seabird and little penguin detection rates across groups of samples, rather than sample-by-sample; (ii) we, ~~using hard-part and genetic analyses. Secondly, we~~ investigate the diversity and relative importance of seabirds consumed by long-nosed fur seals; and (iii) ~~. Finally from two geographic regions: near the center of the long-nosed fur seals' geographic range in western Bass Strait; and at the species' north-eastern range edge in eastern Bass Strait and along the Tasman Sea. Ultimately,U~~and ~~ultimately~~we ~~we~~ explore~~provide~~ a minimum estimate of penguin abundance consumed ~~by long-nosed fur seals~~ by analyzing mitochondrial haplotype diversity among little penguin DNA obtained.

**Methods** ~~1322 words~~

~~*Collections of long-nosed fur seal scats across southeastern Australia*~~*Sample collection*

Individual predator scat samples (n = 99) were collected across multiple time points from four long-nosed fur seal breeding colonies in Bass Strait and NSW,~~in~~ southeastern Australia

(Fig. 1~~, &~~ 2~~a & 2b).~~ ).~~Long-nosed fur seals have only recently begun breeding in Bass Strait and NSW.~~ Pup abundances are illustrated as a conventional proxy for relative seal population (Fig. 1; Appendix S1.1). Most samples were collected from the two larger colonies, Barunguba and Cape Bridgewater, in spring (September) 2016 and summer (January) 2017, with additional samples ~~taken in~~included from spring 2015 and summer 2016 at Cape Bridgewater ~~(Fig. 1)~~. Samples from Gabo Island were collected from ~~one season,~~ summer 2017 ~~(Fig. 1)~~. ~~Additionally, O~~one sample was opportunistically collected from Deen Maar Island and included in assays ~~(Fig. 1)~~. Sample sizes ~~used~~ resulted from balancing adequate replication per site with availability of fresh samples ~~and the costs of genetic analyses~~.

Whole and fresh (~~soft and moist,~~ ~~and therefore~~ < 48 ~~hr~~ h old) ~~faecal~~ scats~~samples~~ were ~~collected~~ sampled to minimise bias from differential DNA degradation ~~of DNA~~ or partial loss of ~~scat~~ material~~, and placed in an air-tight, zip-lock bag~~. Whole scats were thoroughly mixed with individual disposable spatulas at point of collection and a 2 mL subsample was taken from each field-homogenized scat for DNA-based analyses of prey remains. The remaining ~~W~~whole scats were ~~used~~collected for ~~hard-part~~ analyses of morphological prey remains~~,and placed in an~~ using individual ~~air-tight~~, zip-lock bags.~~ Whole scats were used for hard-part analyses of morphological prey remains.~~ ~~Subsamples (2 mL) were taken from whole scats directly at the point of collection in the field for DNA-based analyses of prey remains, by homogenising whole scats with individual disposable spatulas and storing in 2 mL in Eppendorf tubes.~~ Samples were stored within hours of collection between -10˚ and -20˚C in portable freezers (WAECO) for up to 7 d in the field and transferred to -20˚C freezer facilities.

~~*Morphological identification*~~*Identification of seabird morphological and genetic remains ~~in~~ ~~long-nosed fur seal scats~~*

~~Seabird morphological remains are conspicuous across long-nosed fur seal colonies in southeastern Australia (Fig. 2c).~~ All prey items were identified from hard~~-~~ parts using methods described by ~~Kirkwood et al. (2008) and~~ Page et al. (2005). ~~Data on diet items other than birds are the subject of a broader investigation on long-nosed fur seal diet across southeastern Australia.~~ Birds were identified using feathers and other remains such as feet, ~~flippers~~flippers, and heads (Fig. 2~~d~~, Appendix S1.2).

~~*DNA metabarcoding of seabird genetic material from long-nosed fur seal scats*~~

Prey DNA extractions used 250 mg of faecal subsamples and MoBio PowerSoil® DNA Isolation Kits (www.mobio.com) with modifications to the manufacturer's instructions made in response to extraction optimisation (Appendix S1.3). DNA was eluted in 10 mM Tris buffer, MoBio PowerSoil® C6 solution, (www.mobio.com) and stored at -20˚C. Nuclear DNA for positive controls was extracted from muscle tissue (25 mg) of a domestic chicken (*Gallus gallus domesticus*)~~,~~ and a little penguin~~. DNA was extracted from~~ using ~~muscle tissue~~

~~from the centre of the birds' tissue matrix (25mg)~~ with Bioline Isolate II Genomic DNA Kits (~~https://~~www.bioline.com/us/) as per manufacturer instructions.

~~A~~ In total ~~of,~~ 99 faecal DNA sample extracts at two DNA concentrations (neat and 1:10 dilutions), as well as extraction blanks (n = 5), PCR blanks (n = ~~2~~4), and positive controls (n = 2) were screened in duplicates by diagnostic endpoint PCR (dPCR) using the Bird12sa/h assay (Cooper, 1994) (Table S1 ~~&and~~ S2, Appendix S1). ~~The~~ Duplicate dPCR products were run on 1.5% agarose gels to determine the presence/absence of amplified target bird DNA in each duplicate. A total of 32 samples showed target amplicons in both or one duplicate, but not in the extraction ~~blanks~~ and PCR ~~blanks (negative~~ controls~~)~~. ~~We proceeded with sequencing using a single sample of DNA extract (neat concentration) for~~ We sequenced each of the 32 samples that tested positive for birds, and ~~we included~~ two extraction blanks and one positive control (n = 35 samples for sequencing). A single-step fusion tagging PCR procedure was used to attach and ~~.Target samples and controls were each~~ assig~~ned a~~ unique MID (Multiplex IDentifier) tag combination~~s~~, ~~combined with~~ next generation sequencing (NGS) adaptors and the Bird12sa/h assay ~~using a single-step fusion tagging PCR procedure~~. The sequencing workflow~~, including –~~ single-step fusion PCR (Appendix S1), library build, sequencing (150 bp paired-end Illumina Miseq: v2 Nano 150 bp) and demultiplexing~~, –~~ was performed by ~~the~~ Ramaciotti Centre for Genomics, ~~laboratories at the~~ University of New South Wales.

Our bThe bBBioinformatics and sequence quality filtering procedures are described in reproducible detail in reproducible detail in Appendix S1.3. We used Geneious R8.1.5 (Kearse et al., 2012) for to process merge theing paired-end sequences (2x ~150 bp fragments, with overlap of 70 bp) and to identifyretain only those sequences with exact removing matchedflanking sequences – with matching ;-geneticMID tags,s and primers and adapters were retained for each sample. PGenetic primers, adapters and tags were then removed after this initial filtering step to leave the complete target sequences in each sample. These arget sequences were quality controlledfiltered and clustered into molecular operational taxonomic units (OTUs) using the *UPARSE* algorithm and custom bioinformatics pipeline primarily performed in *USEARCH* (Edgar, 2010; Edgar & Flyvbjerg, 2015). Firstly, IL

Notably, through this bioinformatics pipeline, low abundance sequences (are were discarded below expected a threshold of 1%abundances accounting for sequencing platform error (threshold value: < 1% of the total number abundance of all unique sequences) were removed to mitigatereduce the occurrence of sequencing platform error and chimeras), and secondly sequences are were then clustered using a 97% similarity criterion (similar to Berry et al., 2017). A total ofThus 7370 unique seabird DNA sequences, representing a total of

64,700 disaggregated bird sequences, were parsed ~~to the~~to standard sequence filtering and OTU clustering pipeline (with cluster size threshold value of 73)~~.~~, resulting in 47,478 filtered sequences across 99 samples, and these were clustered to 5 OTUs. ~~5 OTUs containing Effectively, a total of 64,700 disaggregated bird sequences were then filtered down to 35,424 sequences across all 99 samples and were subsequently assigned to five unique taxa.~~

Consensus sequences for each OTU were queried against the National Center for Biotechnology Information's (NCBI) GenBank nucleotide database using the algorithm BLASTn (Basic Local Alignment Search Tool) (Benson et al., 2005). The resulting 'blasted' sequences were ~~then~~ assigned to taxa, following criteria and taxonomic reference databases outlined in Hardy et al. (2017) ~~and Deagle et al. (2009)~~ and Appendix S1.3 (Table S3). These ~~objective of these~~ criteria ~~was to ensure~~ maximisedmum confidence in making a taxonomic identification~~, and~~ and ~~a~~ minimisedum~~,~~ while ~~minimise~~ minimising the risk of false positives.

*Haplotype polymorphism analysis ~~and assessment of penguin abundance consumed~~*

~~Due to their high cultural and conservation value, We~~ Wwe sought to ~~to report~~ ~~on~~identifidentifyed~~y~~ ~~the~~ a minimum number of individual little penguin by exploring mtDNA

haplotypes from ~~the (~~12S rRNA~~).~~ sequences obtained. While ~~highly~~ dependent on sequence fidelity, such approaches have been used to explore ~~intrspecific~~intraspecific diversity (similar to Seersholm et al., 2018)~~ metabarcoding data to assist in estimating~~estimate ~~the minimum number of penguins that could have been consumed within samples assayed (similar to Seersholm et al., 2018)~~. ~~Acordinbgl~~W~~y~~~~Accordingly, w~~We imported the quality-filtered file containing 47,478 ~~(quality )~~seabird DNA sequences, produced just prior to OTU~~UT~~ clustering, we disaggregated and matched these sequences in relation to sample identifier and formed these into clusters of unique sequences in Geneious. From the six samples that contained abundant penguin DNA ~~For each sample that contained abundant penguin DNA(~~~~(n = 6,~~ Table S4), we selected ~~only 9~~the nine most abundant unique sequences. Each of these represents~~senting each~~ a sequence abundance of greater than ~~>~~ 7.5~~1~~% of the total sequence abundance of the sample (Table S5). ~~For To enable effective haplotype analysis, we formed the quality-filtered sequences into clusters of unique sequences instead of OTUs and selected only the most abundant representative sequences from each of 10 the 10 samples that tested positive for penguins, excluding samples containing only trace amounts of DNA.~~ This process was ~~conservating in ing~~used to exclude beyond reasonable doubt, any further ~~thousands of~~ sequences that could be attributed to sequencing error. ~~(~~’s ~~ina pattern of error~~

**Commented [MB3]:** and

Stat paper…. There are others too

**Commented [MOU4]:** Perhaps a table indicating the cutoff for each sample, or showing the number of sequences used and those discarded. Have a look and see what you think ☺

**Commented [RM5]:** Technically this is a result – but up to you if you think we need it here.

~~that shows sequences with a single base pair change at an abundance of around 1% of the parent sequence; Berry, T. E., unpublished d~~(Tina – this section is a bit wordy as is).

We produced a minimum spanning haplotype network using the software *PopART* (Leigh & Bryant, 2015) from an alignment of these ~~12~~nine penguin sequences ~~(n = 12 sequences, from n = 10 samples)~~ to~~.~~ ~~This enabled~~ visualisezation ~~of the~~ relationships between haplotypes consumed, ~~their abundances within~~ and between samples ~~and~~ from the different locations ~~where their predators' scats were~~ sampled. All ~~10~~ samples containing little penguin DNA (n = 10) were ~~subsequently~~ then searched for the presence of ~~dominant haplotypes~~ identified haplotypes~~, in order to report on the genetic diversity consumed by long-nosed fur seals, both within and across samples. Thus, .~~ ~~we~~ We ~~estimate~~estimated the number of penguins likely consumed based on the number of ~~how many of the identified penguin~~ haplotype~~s~~ within each ~~were then found in each of the 10 scat~~ sample~~s that were positive for penguin DNA, across geographically and temporally separated samples~~. ~~Logically, two distinct mtDNA haplotypes (12s rRNA) found within a sample corresponded to two distinct birds consumed.~~ Additionally, as samples were collected across multiple days from each location and sampling time, we ~~treat~~treated each sample ~~to be~~as from distinct predators or predation events.

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*Statistical analyses*

~~To compare the detection of seabirds and specifically little penguins using different dietary analysis techniques, whilst~~ To account~~ing~~ for different sampling times and locations, samples were assigned one of seven unique grouping factors that combined location and time (~~e.g.~~e.g., Barunguba, January 2017). The single scat sample collected from Deen Maar Island was not included in statistical tests ~~(n = 1)~~, but ~~seabird remains~~results were reported for future comparisons. The overall detection rates of seabirds and specifically little penguins using different dietary analysis techniques (hard-part vs. DNA; Table S6, Appendix S2) ~~Two~~ were examined using two generalised linear models (GLMs), ~~were~~ constructed in the ~~base~~ *stats* package in R version 4.0.3 (R Core Team, 2020) ~~to examine the detection of both seabirds and penguins, in relation to the methods of dietary analysis (hard part vs. DNA; Table S3, Appendix S2)~~. ~~The~~ A binomial error distribution for presence-absence data was used and ~~an~~ additive term included to ~~examine the effect of~~account for long-nosed fur seal ~~sampling~~ group (location and time). Model fit was assessed using deviance explained and variable significance ($p < 0.05$).

290

291 **Results** ~~1027 words~~

292

293 Overall, the detection rates of seabirds were statistically similar ~~using both~~for each method~~s~~,

294 ~~the morphological identification of prey hard part~~hard-parts ~~and the DNA metabarcoding~~

295 ~~technique, for~~ examining ~~predator diet analysis from scat samples (n = 99;~~ (Fig. 3 ~~& S1~~,

296 Table S3). However, DNA metabarcoding offered additional information: (i) absolute and

297 relative abundance information for amounts of DNA recovered (Fig. S1 & S2 ~~& S3~~, Table

298 ~~S5~~S4), (ii) improved sensitivity in detecting multiple prey taxa within a single scat ~~sample~~

299 (Fig. 4), and (iii) ~~identification of genetic diversity~~haplotypesexploration of little penguin

300 12S~~s~~ rRNA genetic diversity enabled the estimation a minimum number of ~~ing estimation~~

301 ~~of~~predated penguins to the be estimated ~~abundances consumed~~ (Fig. 5, Table S5).

302

303 *Comparing ~~seabird detections using~~ diagnostic ~~hard part~~hard-part and genetic ~~analyses~~ assays*

304

305 Seabirds were detected in 29.3% (n = 29) of samples using diagnostic hard-parts, and 21.2%

306 (n = 21) of samples using DNA metabarcoding (Fig. 3a). Most of these detections were little

penguins, detected in 25.3% (n = 25) of samples with hard-parts and 10.1% (n = 10) of samples with DNA (Fig. 3b). Of these positive detections obtained after quality control and filtering, t~~T~~he majority ~~(> 99%)~~ of DNA sequences for ~~each~~ all seabirds taxa~~on~~ were identified in ~~just 9% (n = 9) of~~9 out of 21 ~~those~~ samples, ~~(Fig. 3a) using both a conventional and stringent standard of quality filtering and cleaning protocols (Appendix S1.3)~~and for little penguins in 6 out of 10 samples~~. The other 12 samples contained low amounts (< 1%) of DNA, calculated~~ relative to the total ~~abundance of~~ DNA obtained for each taxon (Figs 3 & S1, Table ~~S5~~S4), and providing a lower conservative estimate for predation incidence.~~ The~~ same ~~Seabirds~~ seabird taxa were detected by ~~both methods~~ simultaneously in ~~only 10%~~half the positive samples (n = 10)~~ of samples (Fig. 3a)~~, and ~~5% (n = 5) samples~~half of these (n = 5) contained both ~~both~~ little penguin hard-parts and DNA (Fig. 3~~b~~). ~~The other~~Five ~~5~~ samples contained DNA and hard-parts that did not belong to the same seabird taxon~~, likely because these methods measure occurrences based on completely different tissues with different passage times~~ (Figs~~. 3 &~~ 4), and the remaining positive samples from each method represent a detection made by one method alone (n = 6 for DNA, and n = 13 for hard-parts). ~~While~~ t~~T~~he combined proportion of samples ~~containing either diagnostic hard-parts or DNA~~that were positive for ~~from~~ seabirds ~~, or both, amounted to~~was 40% (n = 40), and 30% for little

penguins (n = 30) ~~(Figs. S1a), these dietary analysis methods represent two quasi-independent assays and we argue, are therefore not additive~~.

Mean detection rates were statistically similar for both methods for seabirds and penguins (Table S6). There was greater variability in detection rates across locations using hard-parts compared to DNA (Fig. S3). Minor, albeit statistically significant, differences were observed across sampling groups for seabird detection rates (Fig. S3a, Table S3), but not for penguins (Fig. S3b) (GLM binomial seabird detection ~ location: p-value = 0.017; Table S3). Detection variability was high across sampling locations, times and methods used (Fig. S3a). Little penguins were detected by hard-parts from Gabo Island samples, however only a few sequences of little penguin DNA were detected at Gabo Island and Deen Maar Island, and these sequences did not pass DNA quality filtering.

*Seabird diversity in long-nosed fur seal diets*

~~The~~ DNA-based metabarcoding ~~technique~~ was more sensitive in detecting taxonomic mixtures ~~of taxa~~ in scat samples compared to hard-part analysis (Fig. 4), with 2 distinct ~~prey~~ seabird taxa detected in 5 samples and a single ~~prey~~ seabird taxon in the remaining 16

samples ~~(Fig. 4b)~~. ~~In contrast, d~~Ndiagnostic ~~prey hard-parts typically corresponded to a single prey~~ seabird ~~species within samples and n~~o samples contained more than one identified bird taxon using ~~this method~~diagnostic hard-parts (Fig. 4a).

Little penguins ~~(*Eudyptula minor*)~~ were the main seabird prey species detected using both ~~morphological (Fig. 4a) and DNA-based~~ analyses (Fig. 4~~b~~)~~, both~~ , and this was reflected in all data from DNA – ~~in terms of~~ frequency of occurrence (Fig. 4~~b~~), ~~in~~ total abundance of sequences (Fig. ~~S2~~S1), and ~~in~~ relative sequence abundance ~~of sequences~~ (Fig. ~~S3~~S2). ~~Across all samples in this study, 25.3% (n = 25) contained penguin hard-parts and 10.1% (n = 10) had DNA detection for little penguins (Figs. 3b & 4b). and~~ . ~~Whilst, the majority (> 99%) of little penguin DNA was obtained from 6% of samples (n = 6) (Fig. 3b & S2).~~

~~This study identified three other distinct seabird taxa using both dietary analysis methods.~~ Morphological analysis revealed two additional taxa: a ~~shearwaters~~ shearwater~~at~~ family ~~level~~ group (Procellaridae spp.) (n = 2 samples), and ~~the~~ Australasian gannet (*Morus serrator*) (n = 1) (Fig. 4). DNA metabarcoding detected abundant DNA from two distinct ~~families of~~ shearwater taxa, also at family-level, taxa in 5% (n = 5) and 9.1% (n = 9) of samples, respectively (Tables ~~S4~~ S3 & ~~S5~~S4, Appendix S2). ~~We also identified t~~Bhe ~~b~~lack-

browed albatross (*Thalassarche melanophris*) and greater crested tern (*Sterna bergii*~~),~~ ) occurred in ~~1~~ one sample apiece (Fig. 4, Tables S3 & S4 ~~S5~~). ~~The combined p~~Parallel use of both DNA metabarcoding and hard-part analysis revealed a greater diversity of taxa than would ~~have been identified by~~ either method alone.

~~Occurrence of seabird prey across southeastern Australia~~

~~Seabirds were detected at all main sampling locations and time points, regardless of the predator samples' coming from a range edge or more geographically central fur seal colony (Fig. S4 & S5). Mean detection rates were statistically similar for hard parts compared to DNA methods for both seabirds in general (GLM seabird detection ~ metric: p-value = 0.648), and penguins (GLM penguin detection ~ metric: p-value = 0.200) (Table S3). Detection rates across locations were more variable using hard-parts, with a greater range in proportion of samples with seabird or penguin detection, compared to DNA (Fig. S4).~~

~~There was a minor, albeit statistically significant, difference across sampling groups in the detection rates of seabirds (Fig. S4a, Table S3), but not for penguins (Fig. S4b) (GLM binomial seabird detection ~ location: p-value = 0.017; Table S3). This result was largely~~

**Commented [RM7]:** What do you mean by "main"? Perhaps name them instead?

**Commented [RM8]:** It's not clear why this would matter, so I would delete

~~driven by higher seabird detection rates at Cape Bridgewater for most sampling groups and methods used, as well as for Barunguba for the summer of January 2017, compared to lower seabird detection rates for Barunguba in the spring of September 2016, and for Gabo Island in the summer of January 2017 (Fig. S4a).~~

~~Whilst little penguins account for most of the seabird detections across time and location sampled, large amounts of DNA from both Procellarid spp. (sp1 & sp2) and the black-browed albatross were detected alongside abundant little penguin DNA at Cape Bridgewater and Barunguba (Fig. S2). Trace amounts of little penguin DNA were detected at Gabo Island and Deen Maar Island,Island; however, these sequences did not pass DNA quality filtering procedures. Thus conservatively, we would report that whilst penguins were detected from morphological remains morphologically in scats from Gabo Island, we have not yet reliably detected penguin predation by long-nosed fur seals there or at Deen Maar Island using DNA.~~

*Towards quantifying little penguin consumption*

391 AFrom the 10six6 long-nosed fur seal scat samples that contained suitable levels

392 ofcontaining abundant penguin DNA-,A a total of fiveseven7 little penguin mtDNA

393 haplotypes were identified in fromin samples from Cape Bridgewater (Hhaplotypes 1–2 and

394 42– 4, 6 & 75) and Barunguba (Hhaplotypes 1– 1 & 53)- long-nosed fur seal diets (Fig. 5a),).

395 This was, based on selection of the 12 most abundant unique sequences of penguin DNA

396 within samples. All 10 samplesAll After testing for the unique 7 mtDNA haplotypes across

397 the , oOnly Hhaplotype 53 was unique to Barungubau, the remaining sixfive haplotypes were

398 detected at both sites (Figs. 5b). Hand haplotype 1 was identified three different single

399 sources of DNA from penguins from southeastern Australia (see reference material in

400 Appendix S1.4)our positive control —. containing little penguin DNA were subsequently

401 searched for the presence of these 7 mtDNA haplotypes. Thus whilst 2 haplotypes were

402 detected as being from Barunguba samples, we dido detect additional haplotypes when

403 searching those same samples for haplotypes that could come from elsewhereidentified in

404 other samples (Figs. 5b). Of theseAcross all ten10 penguin positive scat samples, fivesix of

405 these5 contained a single haplotype, whilst the remaining fivefour5 contained between 2–6 4

406 individual mtDNA haplotypes or individual penguins (Fig. 5b). Logically,Taking two distinct

407 genetic haplotypes present within a sample, to represent at least two distinct individual birds

consumed. Thus, we estimate posit at least 21 16 individual penguins were consumed across the all 99 scat samples, from two sampling locations and multiple seasons.

## Discussion 1746 words

Conflicts between iconic species are likely to increase with mounting human pressures on wildlife during the Anthropocene and Validating and applying modern surveillance tools, such as using DNA screeningmetabarcoding, tocan inform decision making as increasingly complex conservation scenarios can better inform decision making, also providing transferable arise.

We leveraged recent advances in cost-effective genetic assaying tools, combined appliedmetabarcoding alongsidewith traditional diet analysis methods,. We contributed the following to investigate a growingwildlife conflict in southeastern Australia. towardssignificant advances in understanding complex predator-prey dynamics both within our local context and to for the broader conservation biology community: . Using the little penguin and long-nosed fur seal predator-prey model, we developed (i) a multi-assay method for comparison of target species identification to producing produce a more reliable

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~~prevalence than that offered by the traditional assay alone; (ii) a reproducible protocol for DNA metabarcoding analyses for identifying target prey species from predator scat samples; and, (iii) an applied haplotype polymorphism analysis for genetic diversity and probable abundances of target species within and between samples using shorter base-pair target DNA. Our analytical framework is reproducible and can be tailored to a broad range of wildlife interaction surveillance efforts. In our study system, this analysis provided key information to conservation practitioners for assessing an emerging wildlife conflict in Australian waters and to determine the next steps in monitoring and managing this conflict.~~

~~Specifically, w~~We provide ~~conservation practitioners with a~~an updated and improved predation prevalence range for seabirds overall (9–29%) and little penguins (6–25%) in ~~the diets of~~ long-nosed fur seal diet~~s in southeastern Australia~~using. We also confirm that little penguins ~~are currently~~remain the ~~most commonly consumed~~most frequently consumed seabird by long-nosed fur seals in comparison to other ~~seabirds~~ avian taxa. Here, ~~(e.g., procellarids, black-browed albatross, greater crested tern, and Australasian gannet).~~ DNA metabarcoding also offered key advantages over morphological analysis – quantitative information on: (i) absolute and relative abundances of ~~DNA~~taxa recovered using DNA, (ii) detection of multiple prey taxa within a single scat sample, and (iii) ~~genetic diversity enabling~~

estimates ~~tionof~~ at least 16 penguins ~~abundances consumed accounting for at least 16 penguins consumed across samples~~by exploring the spatial and temporal distribution of haplotypes.

~~Whilst p~~Previous studies using ~~both~~either assay techniques have identified little penguin remains at relatively low frequencies in ~~samples overall~~relation to seals' total diets (5.9% ~~of samples~~ in Page et al. 2005, <2% in Hardy et al. 2017, ~13% ~~of samples~~ in Goldsworthy et al. 2019). The lower range of estimates observed here (9% of samples for seabirds, 6% for penguins) and based on samples containing large quantities of prey DNA corroborate these previously reported predation rates for the region. ~~,~~ ~~t~~However, the upper range of estimates observed in this study (10% and 25% of samples, respectively for DNA and hard-parts~~25% of samples~~) and recent crashes in little penguin colonies (R. R. McIntosh, pers. comm.) signal~~s~~ a need for broader and increased monitoring of predation mortality~~.~~ ~~Specifically, longer-term and comprehensive sampling programs are needed to further quantify and update the spatiotemporal patterns in consumption by long-nosed fur seals consumption. Little penguin consumption may be more prevalent at certain locations near the centre of their range and patterns in seabird and little penguin consumption may change ,~~ ~~particularly since long-nosed fur seal abundance is expected to increase across south-eastern~~

~~Australia (Shaughnessy et al. 2015)~~~~over time, with changing predator demography through~~ ~~population recovery and~~ ~~through climate change~~. P~~Further~~Furthermore, ~~it may be that a learned behaviour becomes advantageous to a sub-population and is~~~~this~~ predatory behaviours could be transmitted to other predator populations, particularly in response to ~~environmental changes and~~ food web disruption under ocean warming and changes in prey availability and this could have cascading effects on penguin populations. Analysis of the predator's total diet ~~consumed~~ is also warranted to gauge the relative importance of ~~the~~ different prey items~~, , in addition to or in combination with focusing on a specific taxonomic group such as~~~~including~~besides seabirds.~~–~~

While both metabarcoding and morphological methods provided statistically similar results, we emphasize that these represent complementary but quasi-independent assays of predation. Different detection rates reported between these methods in this study are also common across a broad range of predator and prey taxa (Tollit et al., 2009; Zarzoso-Lacoste et al., 2013). Many methodological and biological factors differentially affect the recovery of diagnostic genetic and morphological materials. In another pinniped species, prey soft tissues had a gut passage rate of 48 hours and up to 7 days for hard-parts (Tollit et al., 2009). Soft tissues may contain greater DNA quantities and concentrations, particularly mitochondrial

**Commented [NB10]:** I don't disagree but this comes out of nowhere a little

(Mumma et al., 2016; Granquist et al., 2018), and could be preferentially amplified compared to DNA available from hard-parts, (i.e., feather, fur, or bone). DNA from these tissues is of poor quality and requires a different process for extraction (McDonald & Griffith, 2011; Rothe & Nagy, 2016). Typically, studies report higher detection rates from genetic assays compared to morphological analyses (Egeter et al., 2015; Mumma et al., 2016), but the lower detection rates here are consistent with other pinniped studies (Tollit et al., 2009) and with over-representation of feathers across fur seal scats (Goldsworthy et al., 2019), explaining the higher detection rate from these hard-parts.

~~The observation of v~~Distinct variation in ~~the~~ penguin metabarcodes obtained, beyond that attributed to sequencing error, led us to explore how many individuals might be ~~contained~~ within the data (similar to Seersholm et al., 2018 and Tsuji et al., 2020). While not the ideal locus to explore intra-species diversity,~~Based on haplotype polymorphism,~~ we nonetheless identified 5 haplotypes consumed~~which, when explored temporally and spatially~~, across time and space this ~~determined that~~suggested~~-~~ at least 16 penguins were consumed~~, present in 10 of the 99 long-nosed fur seal scat samples that were positive for penguin DNA and passed stringent sequence filtering procedures~~. This is very likely an underestimate of predation, firstly because we~~-~~ used~~used a relatively conserved mitochondrial gene~~ the highly conserved gene for 12S ribosomal RNA ~~which is conserved~~(Banks et al., 2002)~~, and recovered ~230 bp DNA fragments~~, selected for proven reliability in detecting seabirds (Hardy et al. 2017). Targeting alternative markers (~~–~~variable ~~longer~~ barcodes or

microsatellites)- could reveal greater genetic diversity, however we did not succeed with a COI barcode (Appendix S1.2). T. Secondly, to avoid interpreting error (PCR or sequencing) as separate individuals we followed stringent sequence quality and abundance filtering and we were extremely selective for representative haplotype sequences within samples to exclude the possibility of false positives beyond sequencing error, and we likely excluded several low DNA-abundance, true positives ( and reasonable doubtDeagle et al., 2013).

In contrast, lsMost scats containingwith penguin DNA contained a single haplotypessingle haplotype, however some ifour scats (N=???? )could contained up to 4 distinct genetic haplotypes, indicatingsuggesting that long-nosed fur seals can consume multiple penguins in a single foraging trip or within 48h of sampling. This result provides practitioners with a significantly more reliable method of estimating predation incidence and impact than previously when it was methods constrained to assumedptionsing that each scat containing feathers corresponded to a single consumed bird (Page et al., 2005, Mumma et al. 2016). If little penguin predation becomes an important individual foraging strategy even for some long-nosed fur seals, this could have serious negative impacts for isolated penguin populations. DNA-based methods are transferable across systems and offer vast potential for technological and methodological improvements (Tsuji et al., 2020) over traditional morphological diet assays.

Quantifying predation can be difficult for certain taxa and current DNA-based tools already offer significant advantages over identifications of morphological prey remains., particularly for cartilaginous or gelatinous taxa. DNA analyses can detect larger prey missed in hard-parts analyses: for example, fur seals may break apart and selectively eat larger prey. Many predators often process large, feathered prey differently than they do smaller prey that can be swallowed whole – fur seals thrash seabirds into pieces or tearremoveseabird their skin and feathers offprior to consumption (Hocking et al., 2016))and regurgitate large prey remains (Hocking et al., 2016; Page et al. 2005; Mumma et al., 2016)terrestrial have been reported to . Morphological analyses of f faeces can be problematics they are . Fur seals also to the exclusion of diagnostic remains (-Hard-part analysis typically assigns one individual to remains such as a pair of fish otoliths, a bird skull, paired feet wings or feet or and paired upper and with lower cephalopod beaks, however. Hh, assigning the number of individuals to remains such as feathers or fur has been simplistic and could over-represent predation by, for example, counting the presence of feathers in a single scat as one bird (Page et al., 2005). AA To better quantify seabird predation, a recent controlled feeding trial identified that the morphological remains of a single penguin could appear in up to 5 five separate fur seal scats on average (Goldsworthy et al. 2019).Fur seals are also known to regurgitate large prey remains such as beaks, feathers, heads, and flippers highlighting issues with what samples to

use for morphological analyses with this predator (R. McIntosh pers. obs.). Additionally, recent scat clearing and re-sampling experiments indicated that penguin feathers, present in fur seal scats, may persist in the environment longer than finer particles (e.g., fish otoliths) (S-L Reinhold, unpublished data) , likely resulting adding to the in an overestimation of those taxapenguins in diet analyses and overestimation of their consumption.when using morphological remains from old and fresh scats

The DNA analysis supports this hypothesis. While seabirds were detected in 29 samples by hard-part analysis, whileseabird DNA was only detected in 21 samples and simultaneous detection occurred in only 10 samples, providing further evidence that the methods applied are parallel and more informative together, but not additive. Methodological and biological factors differentially affect the detection rates for genetic and morphological assays within a sample, as well as the likelihood for soft and hard prey remains to co-occur within the same samples. For example, soft tissues may have a gut passage rate of 48 hours compared to hard-parts that may be retained for up to 7 days (Tollit et al., 2009). DNA found in the amorphous, soft parts of an animal may be more easily amplified even in a degraded and digested sample matrix such as scats, compared to DNA available from hard-parts, feather, fur or bone (Mumma et al., 2016; Granquist et al., 2018). One can extract DNA from

the latter chitinised or keratinized tissues; however, being of poor quality, this typically requires a different process than amorphous material (McDonald & Griffith, 2011, Zarzoso-Lacoste et al., 2013; Rothe & Nagy, 2016). Additionally, morphological remains such as feathers may be over-represented across fur seal scats regardless of the method used to identify the taxon (S L Reinhold, unpublished data; Casper et al., 2007a; Tollit et al., 2009). comparing DNA and hard-parts analyses analysesin DNA methods detected in our study have also beenreportedother , likely due to the reasons outlined previously(Tollit et al., 2009).S followed. Wand .SThis could be for a couple of reasons: Firstly, DNA found in the soft parts of an animal is readily available and easily amplified. C. However, as these soft parts age, and are digested, the DNA degrades. So, in many cases, while the hard parts take longer to pass after a meal and can be seen for longer, the soft parts have already passed through the digestive tract and any remains are unlikely to be detected using eDNA methodologies. The extraction of DNA from feather and bone requires a different process than that was used for the scat analysis and the DNA is often of poor quality (McDonald & Griffith, 2011, Rothe & Nagy, 2016).

Secondly, therehere can be some stochasticity in DNA amplification from a sample. This occurrence can be mitigated by the use of replicates from the same sample or the use of

~~individually tagged PCR replicates. It is noteworthy that seabird DNA was amplified in up to 32 samples and sequenced successfully in 25 samples when we included samples with trace amounts of DNA. We chose to follow stringent sequence quality filtering to exclude the possibility of false positives beyond reasonable doubt, even though we did not obtain seabird DNA in our extraction blanks or negative controls, thereby making a false positive unlikely. Deagle et al. (2013) acknowledged that such stringent DNA processing steps would exclude true positives as well as quantitative information on prey consumption. Indeed, several fur seal samples contained trace amounts of DNA and morphological remains of the same taxon (ADD SI DATA), so our simultaneous detection rates of hard parts and DNA is conservative. Additionally, there may be some stochasticity in DNA amplification from a sample (Egeter et al., 2015). We mitigated this by thoroughly homogenizing samples and screening multiple replicates from the same sample, as well as using individually tagged PCR replicates. In future research, genetic methodologies could eclipse hard-parts analyses if genetic techniques can be used to augment morphological identifications by targeting non-identifiable prey tissues (Ford et al., 2011; Méheust et al., 2015).~~

~~Based on haplotype polymorphism, we we propose that determined that at least 21 individual penguins were consumed and occurred in only from the 10% of the 99 long-nosed~~

**Commented [RM11]:** This should come after the previous paragraphs because they are all about the differences in the methods, then you summarise our stringent method here.

**Commented [RM12]:** better to say how many if we can

**Commented [RM13]:** I think you can tell that I don't understand what this means or its intent. Using "unless" to end a paragraph is often not a good way to finish. How does this statement reinforce the main purpose of this paragraph (in green)?

fur seal scat samples. Further that had reliable amounts of penguin DNA from Cape Bridgewater and Barungaba Therefore, the estimated number of penguins eaten (21 individuals) was higher than the prevalence of scats with penguin DNA (10 scats), so assuming that each scat with feathers represents one bird is simplistic and possibly an under-estimate not an over-estimate. Using haplotype detection, a, a single scat could contain up to 6 six haplotypes or individual penguins. We posit expect that this penguin number abundance of 21 individuals is likely an underestimate, firstly due to because ofhighly conserved genetic diversity and limited spatial variability in genetic structuring of little penguins based on microsatellite and mitochondrial DNA assays (Peucker et al., 2009; Burridge et al., 2015; Vardeh, 2015). SecondlyAdditionally, this study uses used a conserved mitochondrial gene, 12S ribosomal RNA, and recovered ~230 bp DNA fragments. This gene was, selected for proven reliability in detecting seabirds (Hardy et al. 2017). Targeting longer and more variable barcodes would likely reveal greater genetic diversity and thus further increase our estimation of individual penguins consumed. Decisions on target genes must be balanced with the fact that faecal DNA is highly degraded and the recovery of longer fragments can be problematic, but at the loss of detecting shorter DNA traces typical in degraded faecal material (Taberlet et al. 2012). If longer fragments are targeted, DNA traces from birds that are more digested may be lost. However, ongoingoO improvements in DNA extraction and sequencing techniques will ensure genetic tools remain at the forefront of wildlife forensics and ecological monitoring.

**Commented [RM14]:** Do you agree?
I think this is the most interesting finding for this haplotype result.

**Commented [RM15]:** Is this needed?

606 ~~Our results show that parallel applications of these two techniques represent nearly~~
607 ~~independent and informative assays: they are not additive or exclusive. This paper~~We
608 ~~ultimately posits~~demonstrate ~~that DNA-based methods will significantly advance wildlife~~
609 ~~conflict surveillance and impact assessment between~~ species of ~~conservation priority species.~~
610 ~~DNA metabarcoding provided key additional information here, critical to assessing predator-~~
611 ~~prey interactions within a wildlife conflict and conservation management context: (i) offering~~
612 ~~multiple metrics in addition to occurrence rates; (ii) detecting multiple prey taxa within a~~
613 ~~single sample; and (iii) identifying genetic diversity enabling~~ to ~~estimation~~ estimate of
614 ~~penguin~~the ~~abundances~~ of~~consumed~~ prey. ~~We recommend the development and optimization~~
615 ~~of cost-effective assays tailored to the needs of specific wildlife conflict scenarios in order~~
616 ~~to~~to ~~better quantify and monitor these interactions. The use of multiple target genes typically~~
617 ~~produces more reliable results with which to form consensus on~~for ~~determining predation~~
618 ~~prevalence and likely impacts. Genetic~~ This ~~method may be extended by including genetic~~
619 ~~screening for~~ to ~~identify predator DNA enables individual predator identifications~~ (Wegge et
620 ~~al., 2012), which~~ and this may~~would~~ ~~be of especial interest to managers~~valuable ~~when~~
621 ~~considering~~ controversial ~~control~~ strategies are on the table for controlling predation. If
622 ~~consumed biomass information is needed, we recommend developing DNA-to-tissue-based~~

**Commented [RM16]:** I think "posit" is used too often in the text. It is a very nice word so let's help it out and save it from being annoying by diversifying ☺

**Commented [RM17]:** The other points are general, so I have made this one general too

~~correction factors (Thomas et al., 2014). Numerous studies have developed species-specific and cost-effective assays using older technology, which and that could be applied to large sample sizes and large numbers of predatory taxa, for the detection of specific taxonomic groups of high conservation or commercial interest (Fox et al., 2012; Hunter et al., 2012; Schreier et al., 2016). For example, Skaala et al. (2014) used genetic techniques not only to identify the prey species of interest, but also used several microsatellite markers to identify the origin of prey stock at high spatial resolution.~~ Finally, our results demonstrate a need for research and development of techniques at the nexus of population genetics and environmental sampling – including screening for predator genetic diversity to identify individuals in a population contributing to predation of a sensitive or valuable species (Wegge et al., 2012), developing species-specific probes using older and cheaper technology (Fox et al., 2012), cross-validation of eDNA data with quantitative PCR (Murray et al. 2011), or development of penguin-specific DNA-to-tissue-based correction factors could provide consumed biomass information (Thomas et al., 2014). ~~metabarcoding and morphological~~

~~Predator P~~Additionally, p~~A final consideration – p~~redator impacts need to be considered and managed within ~~an~~ up-to-date cumulative impact assessments for threats~~, here to . here to~~We have delivered an important step towards this for little penguins in ~~southern~~

south-eastern Australia~~, before money is spent on strategies that may not be effective, such as native predator culling~~. ~~Like~~ Similar to ~~many~~ other wildlife conflict situations, endemic predation is natural~~,~~ and often habitat degradation, environmental change and invasive species are more significant sources of impact to susceptible species (Hervieux et al., 2014; Marshall et al., 2016; Ropert-Coudert et al., 2019). ~~Our results indicate that seabird and particularly little penguin predation may be a relatively~~an important individual foraging strategy for some long-nosed fur seals, with potentially negative impacts for local penguin populations. However, this threat needs to be assessed alongside other impactful and cumulative stressors (e.g. habitat degradation and introduced terrestrial predators) .

~~(Kirkwood et al., 2014).Conflicts between iconic species are likely to increase with mounting human pressures on wildlife during the Anthropocene and surveillance tools such as using DNA screening can inform decision making as increasingly complex conservation scenarios arise.~~

~~It is important to acknowledge that the scale and prevalence of predator-prey interactions may have been altered as a result of anthropogenic-induced changes to both fur seals and penguins over the last 200 years. Accurate estimates of historical seal and penguin populations, and their interactions, are largely unknown to Western science. However, knowledge of pre-colonial systems may be held by Traditional Custodians of the land and sea~~

**Commented [RM18]:** Bring the final sentence back to the start of the introduction – I have had a go at it.

~~country and could provide insight regarding the relationship between the little penguin and the long-nosed fur seal.~~

**Supporting Information**

PINP_Bird_supplement.docx ~~document included in submission~~.

**Data Availability**

Datasets and code used ~~to produce these analyses and figur~~for figures and analyses~~es~~ will be ~~made available via~~placed in ~~an~~ public online ~~data publication~~ repository upon acceptance of this manuscript for publication.

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**Figure Legends**

**Figure 1.** a) Long-nosed fur seal scat collection sites (n = total number of samples~~sampling effort numbered~~)~~.~~, including ~~P~~pup abundances for sampling locations (McIntosh et al., 2014)~~,~~ as an index of seal population ~~, has been included for sampling locations, to illustrate the~~ relative importance of ~~these~~ sites ~~for long-nosed fur seal populations in southeastern Australia~~. Sampled sites were: Cape Bridgewater (38.3013˚ S, 141.4062˚ E) and nearby Deen Maar Island (formerly Lady Julia Percy Island, 38.4161˚ S, 142.0038˚ E) from western Bass Strait, Victoria; Gabo Island in eastern Bass Strait, Victoria (37.5649˚ S, 149.9133˚ E); and Barunguba (formerly known as Montague Island 36.2510˚ S, 150.2270˚ E) at the northeastern breeding range in New South Wales (NSW). ~~Range~~ Species distributions ~~of both species~~ shown for b) long-nosed fur seals and c) little penguins ~~using Atlas of Living Australia distribution data~~ (data from ALA, 2019).

**Figure 2.** ~~Contextual images of~~ a) ~~the l~~A long-nosed fur seal, *Arctocephalus forsteri*, from Barunguba, NSW; b) the little penguin, *Eudyptula minor*, often burrowing near fur seal colonies; ~~and~~ c) and d) ~~examples of~~ seabird remains are conspicuous at long-nosed fur seal haul-outs and colonies,~~in~~ among scats and regurgitatesc~~) and d), often found as regurgitates, from long-nosed fur seal haul-outs and colonies~~.

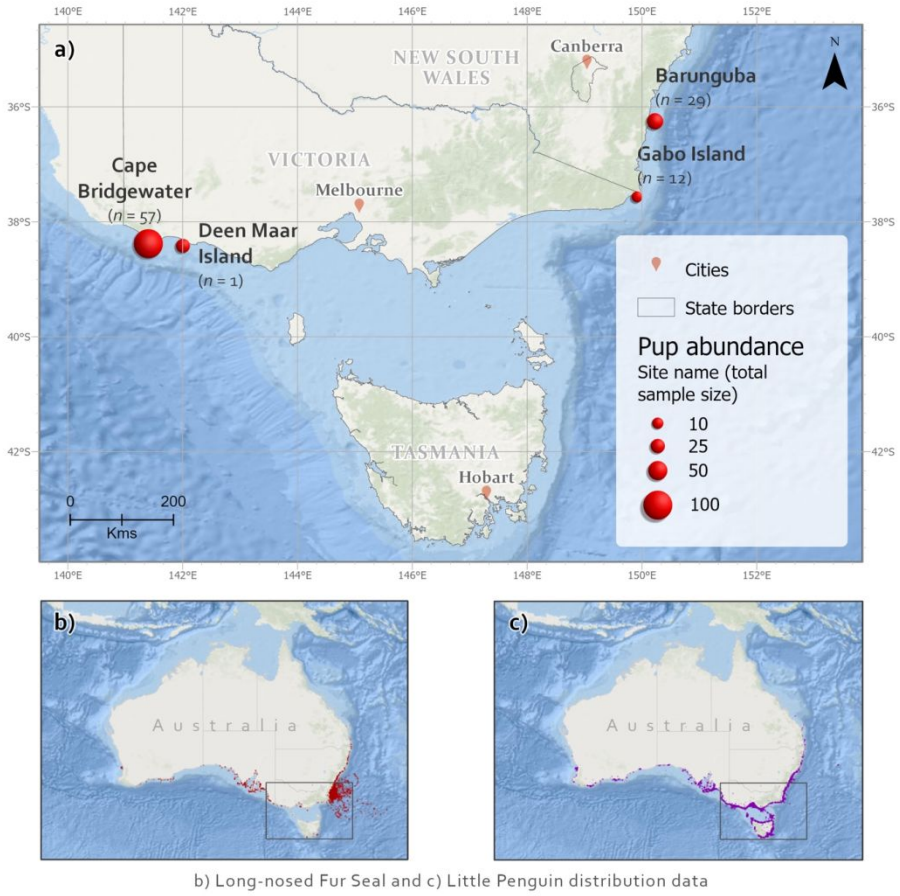
**Figure 3.** Detections ~~across long-nosed fur seal samples~~ of a) seabird and b) little penguin diagnostic hard-parts ('hp') and DNA ('dna'~~dna~~), as a percentage of all long-nosed fur seal samples (n = 99). We report all genetic sequences obtained from standard sequence quality control and filtering~~,~~ 'DNA (all)', ~~as well as~~and for samples that contained large sequence quantities of sequences~~,~~ 'DNA (abundant)' ~~(> 99% of sequences~~ (> 90% of filtered sequences~~filtered after sequence quality filtering~~); and~~. We also illustrate~~ the number of samples that contained both the morphological and genetic remains of the same seabird ('same taxon').

**Figure 4.** The diversity of seabirds ~~taxa~~ identified in long-nosed fur seal samples: a) using hard-part analyses (n = 29) and b) using DNA-based methods (n = 21). GI = Gabo Island. ~~The total (Fig. S2) and relative (Fig. S3) contribution of seabird taxa within samples based on DNA abundance are included in Appendix S2.~~

**Figure 5.** Little penguin genetic diversity (for ~230 bp 12S rRNA gene) a) presented as a minimum spanning network of ~~7~~ five distinct haplotypes, and b) ~~estimated~~ number of ~~individuals~~ haplotypes contained within each penguin-positive sample, ~~consumed across the sample region and time period based on haplotype consumption,~~ including haplotype sequence abundances within samples. ~~Numbers in each circle represent a unique haplotype identifier. Here, each unique haplotype within an individual fur seal scat sample represents an individual penguin consumed (b) and we overlay the genetic sequence abundance identified within samples that tested positive for penguin (n = 10) for each haplotype.~~

961 **Figures ~~& Tables~~**

962




b) Long-nosed Fur Seal and c) Little Penguin distribution data

963

964 **Figure 1.**

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967 **Figure 2.**

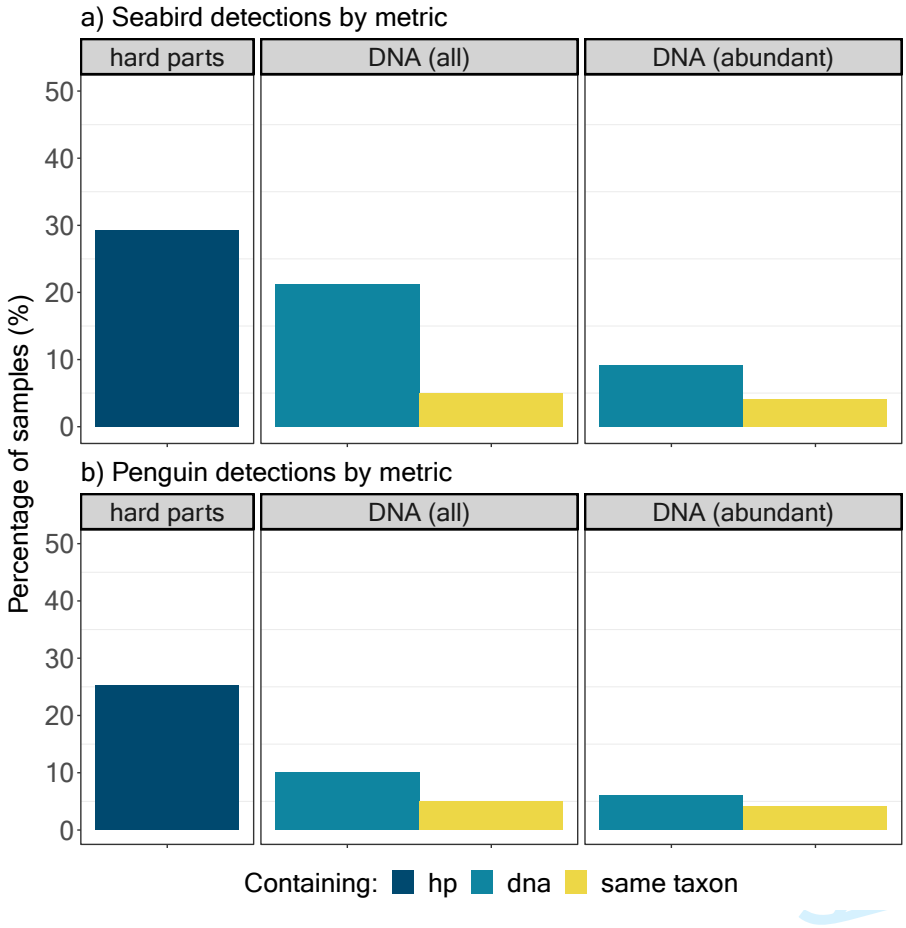

a) Seabird detections by metric
hard parts
DNA (all)
DNA (abundant)
b) Penguin detections by metric
Percentage of samples (%)
0
10
20
30
40
50
Containing: hp dna same taxon

Figure 3.

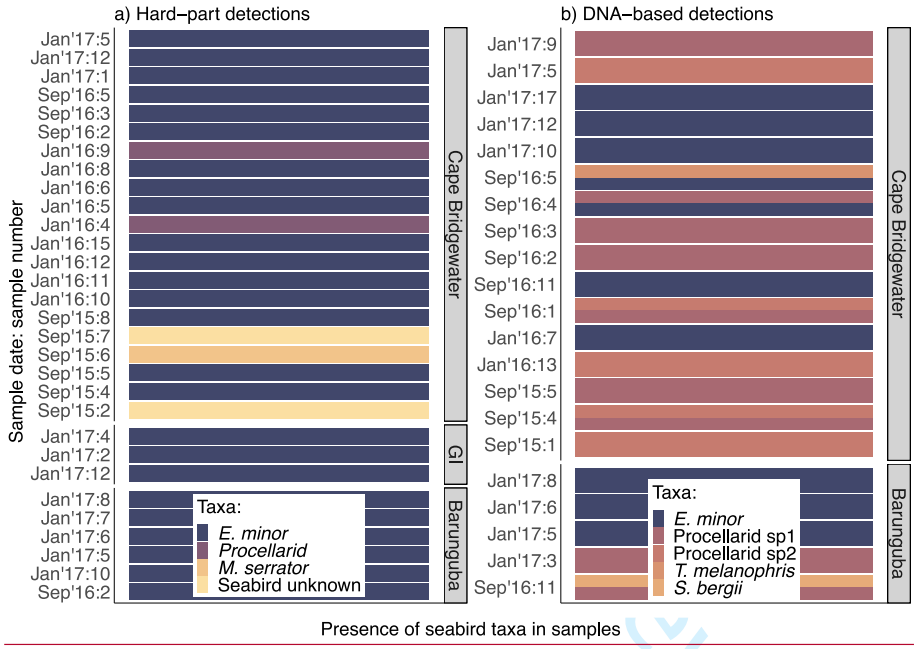
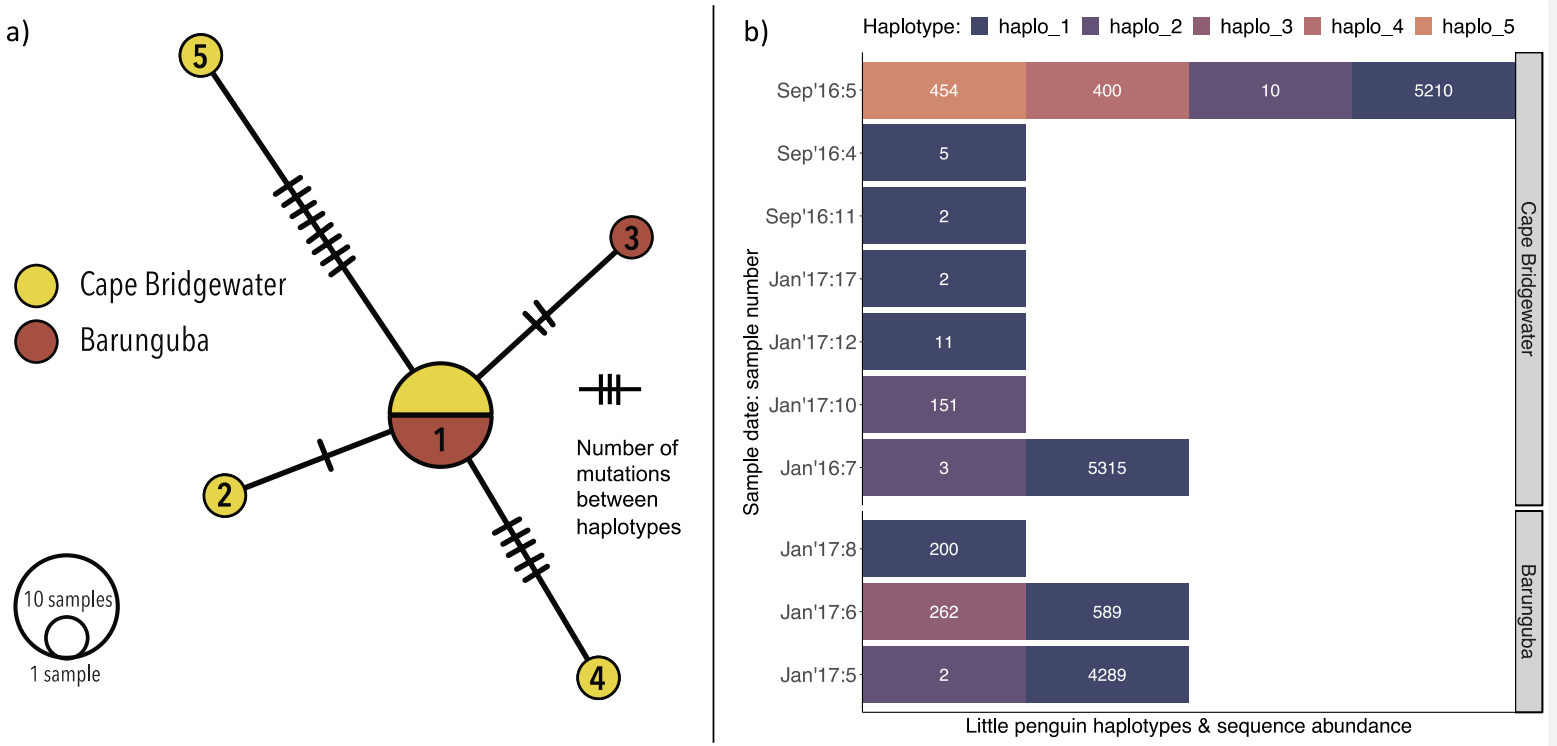



**Figure 4.**


a)
5
3
1
2
4
Cape Bridgewater
Barunguba
Number of mutations between haplotypes
10 samples
1 sample
b)
Haplotype: haplo_1 haplo_2 haplo_3 haplo_4 haplo_5
Sep'16:5
Sep'16:4
Sep'16:11
Jan'17:17
Jan'17:12
Jan'17:10
Jan'16:7
Jan'17:8
Jan'17:6
Jan'17:5
454
400
10
5210
5
2
2
11
151
3
5315
200
262
589
2
4289
Sample date: sample number
Cape Bridgewater
Barunguba
Little penguin haplotypes & sequence abundance

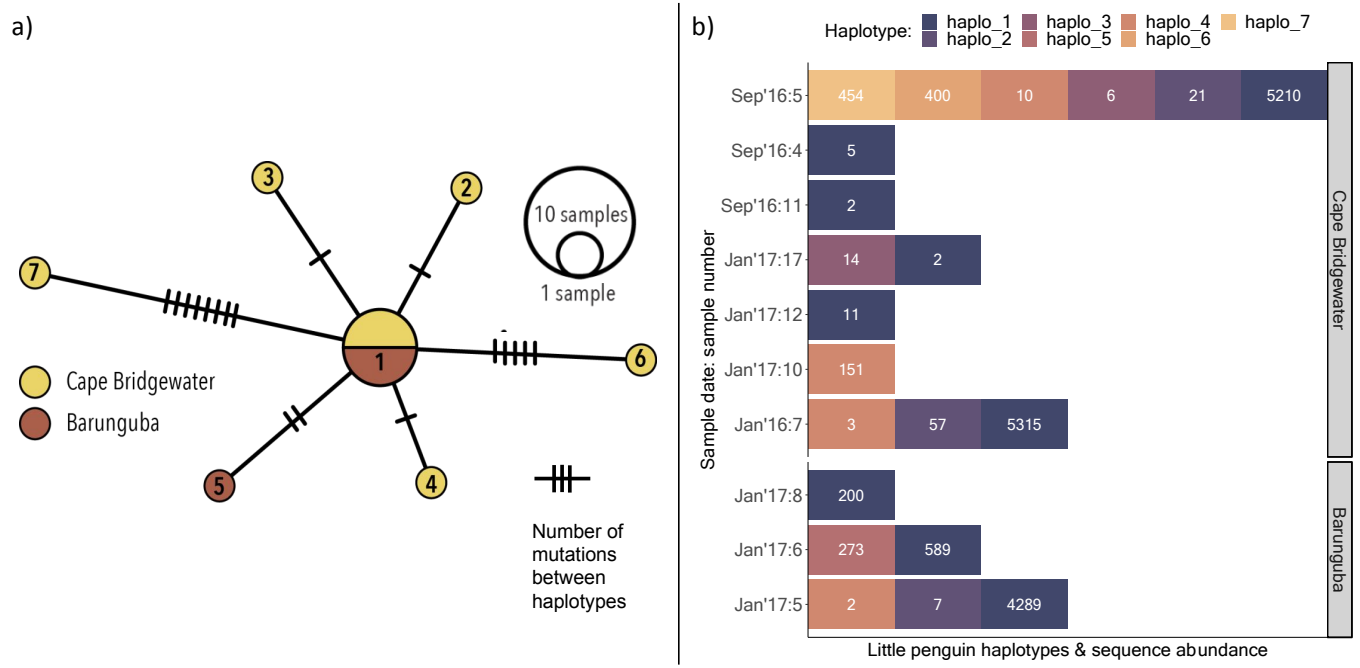

a)
10 samples
1 sample
Cape Bridgewater
Barunguba
Number of mutations between haplotypes
b)
Haplotype:
haplo_1
haplo_2
haplo_3
haplo_5
haplo_4
haplo_6
haplo_7
Sep'16:5
Sep'16:4
Sep'16:11
Jan'17:17
Jan'17:12
Jan'17:10
Jan'16:7
Jan'17:8
Jan'17:6
Jan'17:5
454
400
10
6
21
5210
5
2
14
2
11
151
3
57
5315
200
273
589
2
7
4289
Sample date: sample number
Cape Bridgewater
Barunguba
Little penguin haplotypes & sequence abundance

**Figure 5.**