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Quantifying wildlife conflicts by combining eDNA metabarcoding and traditional diet analysis

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Abstract:	Wildlife conflict 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 mutli-assay framework for quantifying predation broadly across systems and wildlife conflict scenarios. We combine two ecological surveillance techniques applied to predator diet analysis, traditional morphometric (hard-part) and DNA metabarcoding (genetic) analyses, to provide managers with an estimated incidence of predation, the number of species impacted and quantitative information on prey importance to the predator. Further, we perform a polymorphism analysis on obtained prey DNA to estimate 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 the incidence of predation by recovering and protected long-nosed fur seals (Arctocephalus forsteri) of 9–29% for seabirds and 6–25% for the culturally valued little penguin (Eudyptula minor), and higher than previously reported from traditional morphological assays. DNA metabarcoding proved more sensitive in identifying additional seabird prey and provided relative quantitative information where multiple prey species occur within a sample. Polymorphism analysis of consumed little penguin DNA identified distinct mitochondrial haplotypes – representing a minimum of 21 individual penguins consumed across just 10 fur seal scat samples. We recommend broad spatiotemporal sampling of predator diets to further quantify predation incidences and hotspots of concern for wildlife conflict management using the most cost-effective assaying techniques. We highlight the utility of DNA metabarcoding techniques in providing more reliable quantitative information on predation incidence and likely abundance of impacted species of conservation concern.

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Wildlife conflict 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 mutli-assay framework for quantifying predation broadly across systems and wildlife conflict scenarios. We combine two ecological surveillance techniques applied to predator diet analysis, traditional morphometric (hard-part) and DNA metabarcoding (genetic) analyses, to provide managers with an estimated incidence of predation, the number of species impacted and quantitative information on prey importance to the predator. Further, we perform a polymorphism analysis on obtained prey DNA to estimate 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 the incidence of predation by recovering and protected long-nosed fur seals (*Arctocephalus forsteri*) of 9–29% across 6 seabirds, and 6–25% for their main seabird prey – the culturally valued little penguin (*Eudyptula minor*), and higher than previously reported from traditional morphological assays. DNA metabarcoding proved more sensitive in identifying additional seabird prey and provided relative quantitative information where multiple prey species occur within a sample. Polymorphism analysis of consumed little penguin DNA identified distinct mitochondrial haplotypes – representing a minimum of 21 individual penguins consumed across just 10 fur seal scat samples. We recommend broad spatiotemporal sampling of predator diets to further quantify predation incidences and hotspots of concern for wildlife conflict management using the most cost-effective assaying techniques. We highlight the utility of DNA metabarcoding techniques in providing more reliable quantitative information on predation incidence and likely abundance of impacted species of conservation concern.

Introduction

Conflicts between iconic species are likely to increase with mounting human pressures on wildlife during the Anthropocene. New conservation and wildlife management scenarios are emerging 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 (Marshall et al., 2016). 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 et al., 2007); wolves and caribou (Hervieux et al., 2014). The interactions themselves are natural, however they 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, and for effective management of all species concerned (Marshall et al., 2016).

The ultimate goals in investigating the incidence and impacts of predator prey interactions involves determining prey identities, 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

environmental monitoring (Thomsen & Willerslev 2015; Stat et al. 2019) and utility for achieving these goals by: (i) identifying species 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 purposes and sample population estimation (Sigsgaard et al., 2016; Seersholm et al., 2018).

An emerging wildlife conflict in southeastern Australia involves the recovery of long-nosed fur seal (*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 1800's for the fur trade and culling into the 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 pup production occurs. Total estimates of mainland Australian seal populations prior to sealing were never made, however it is

noteworthy that the recent recovery of long-nosed fur seals likely represents a small fraction of their population prior to European colonisation (Ling, 2014).

Little penguins are a popular tourist attraction and locally valued species to communities across southern Australia (Tisdell & Wilson, 2012), with an estimated 470,000 little penguin individuals (BirdLife International, 2021). Yet, 60% of sites have unknown population trends, 29% of colonies are deteriorating and most persist on offshore islands in southern Australia where they 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 et al., 2014), (ii) inscreasing susceptibility to hyperthermia during increasingly frequent terrestrial heat waves (Lauren Tworkowski, La Trobe University, unpublished data), and (iii) large-scale changes to foods web caused by ocean warming and competition with marine fisheries (Ropert-Coudert et al., 2019). Little penguins and other seabirds have been identified in the diets of juveniles, sub-adult and adult male long-nosed fur seals, at two locations in southern Australia and at relatively low frequencies (Page et al. 2005; Hardy et al. 2017; Goldsworthy et al. 2019). However, penguin abundances consumed and predation impacts have been difficult to estimate. 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, 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 previous estimates of penguin biomass consumed, and thus predator impacts were likely overestimated (Goldsworthy et al. 2013).

Both species are federally protected and garner significant cultural and conservation value (*Environment Protection and Biodiversity Conservation Act*, 1975 & 1999). Both are also listed as 'Least Concern' by the IUCN Redlist (IUCN 2020). However, the recovery and protection of many Australian seal species continues to conflict with many 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. Yet, the perception of this conflict has led to persistent and vocal calls to cull the long-nosed fur seal population in South Australia (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.

We combine two ecological surveillance techniques – morphometric (hard-part) and DNA metabarcoding (genetic) assays of long-nosed fur seal scats – to assess predation incidence and potential impact on little penguin and seabirds across southeastern Australia.

First, we aimed to compare seabird and little penguin detection rates using hard-part and genetic analyses. Secondly, we investigate the diversity and relative importance of seabirds consumed by long-nosed fur seals 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, we provide a minimum estimate of penguin abundance consumed by long-nosed fur seals by analyzing mitochondrial haplotype diversity among little penguin DNA obtained.

Methods

Collections of long-nosed fur seal scats across southeastern Australia

Individual predator scat samples (n = 99) were collected across multiple time points from four long-nosed fur seal breeding colonies in southeastern Australia (Fig. 1, 2a & 2b). Long-nosed fur seals have only recently begun breeding in Bass Strait and NSW. Pup abundances are illustrated as a 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 spring 2015

and summer 2016 at Cape Bridgewater (Fig. 1). Samples from Gabo Island were collected from one season, summer 2017 (Fig. 1). Additionally, 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 old) faecal samples were collected to minimise bias from differential degradation of DNA or partial loss of scat material, and placed in an air-tight, zip-lock bag. 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 of seabird 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 and heads (Fig. 2d, Appendix S1.2).

150

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

152

153 DNA extractions used 250 mg of faecal subsamples and MoBio PowerSoil® DNA Isolation
154 Kits (www.mobio.com) with modifications to the manufacturer's instructions made in
155 response to extraction optimisation (Appendix S1.3). DNA was eluted in 10 mM Tris buffer,
156 MoBio PowerSoil® C6 solution, (www.mobio.com) and stored at -20˚C. Nuclear DNA for
157 positive controls was extracted from a domestic chicken (*Gallus gallus domesticus*), and a
158 little penguin. DNA was extracted from muscle tissue from the centre of the birds' tissue
159 matrix (25mg) with Bioline Isolate II Genomic DNA Kits (https://www.bioline.com/us/) as
160 per manufacturer instructions.

161 A total of 99 faecal DNA sample extracts (neat and 1:10 dilutions), as well as
162 extraction blanks (n = 5), PCR blanks (n = 2), and positive controls (n = 2) were screened in
163 duplicates by diagnostic endpoint PCR (dPCR) using the Bird12sa/h assay (Cooper, 1994)
164 (Table S1 and S2, Appendix S1). The dPCR products were run on 1.5% agarose gels to
165 determine the presence/absence of amplified target bird DNA. A total of 32 samples showed
166 target amplicons. Target samples and controls were each assigned a unique MID (Multiplex
167 IDentifier) tag combination, combined with next generation sequencing (NGS) adaptors and
168 the Bird12sa/h assay using a single-step fusion tagging PCR procedure. The sequencing
169 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 bioinformatics and sequence quality filtering procedures are described in reproducible detail in Appendix S1.3. We use Geneious R8.1.5 (Kearse et al., 2012) for processing paired-end sequences and removing genetic tags and primers. Target sequences were clustered into molecular operational taxonomic units (OTUs) using the *UPARSE* algorithm and custom bioinformatics pipeline primarily performed in *USEARCH* (Edgar, 2010; Edgar & Flyvbjerg, 2015).

Notably, through this bioinformatics pipeline, low abundance sequences are discarded below expected threshold abundances accounting for sequencing platform error (threshold value: < 1% of total number of unique sequences), and sequences are clustered using a 97% similarity criterion (similar to Berry et al., 2017; Hardy et al., 2017). A total of 7370 unique seabird DNA sequences were parsed to the standard sequence filtering and OTU clustering pipeline (with cluster size threshold value of 73). 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. The objective of these criteria was to ensure maximum confidence in making a taxonomic identification, and minimise the risk of false positives.

Haplotype analysis and assessment of penguin abundance consumed

We report on individual little penguin mtDNA haplotypes from the (12S rRNA) metabarcoding data to assist in estimating the minimum number of penguins that could have been consumed within samples assayed (similar to Seersholm et al., 2018). For haplotype analysis, we selected the most abundant representative sequences from 10 samples that tested positive for penguins, excluding samples containing only trace amounts of DNA. We produced a minimum spanning haplotype network using the software *PopART* (Leigh & Bryant, 2015) from an alignment of these sequences (n = 12 sequences, from n = 10

203 samples). This enabled visualization of the relationships between haplotypes consumed, their
204 abundances within and between samples and from the different locations where their
205 predators' scats were sampled. All 10 samples containing little penguin DNA were
206 subsequently searched for the presence of dominant haplotypes identified, in order to report
207 on the genetic diversity consumed by long-nosed fur seals, both within and across samples.
208 Thus, we estimate the number of penguins likely consumed based on how many of the
209 identified penguin haplotypes were then found in each of the 10 scat samples that were
210 positive for penguin DNA, across geographically and temporally separated samples.
211 Logically, two distinct mtDNA haplotypes (12s rRNA) found within a sample correspond to
212 two distinct birds consumed. Additionally, as samples were collected across multiple days
213 from each location and sampling time, we treat each sample to be from distinct predators.
214

215 *Statistical analyses*

216

217 To compare the detection of seabirds and specifically little penguins using different dietary
218 analysis techniques, whilst accounting for different sampling times and locations, samples
219 were assigned seven unique grouping factors that combined location and time (e.g.

220 Barunguba, January 2017). The single scat sample collected from Deen Maar Island was not
221 included in statistical tests (n = 1), but seabird remains were reported for future comparisons.
222 Two generalised linear models (GLMs) were constructed in the base *stats* package in R
223 version 4.0.3 (R Core Team, 2020) to examine the detection of both seabirds and penguins, in
224 relation to the methods of dietary analysis (hard part vs. DNA; Table S3, Appendix S2). The
225 binomial distribution for presence-absence data was used and an additive term included to
226 examine the effect of long-nosed fur seal sampling group (location and time). Model fit was
227 assessed using deviance explained and variable significance.

228

229 **Results**

230

231 Overall, the detection rates of seabirds were statistically similar using both methods, the
232 morphological identification of prey hard parts and the DNA metabarcoding technique, for
233 predator diet analysis from scat samples (n = 99; Fig. 3 & S1, Table S3). However, DNA
234 metabarcoding offered additional information: (i) absolute and relative abundance
235 information for amounts of DNA recovered (Fig. S2 & S3, Table S5), (ii) improved
236 sensitivity in detecting multiple prey taxa within a single scat sample (Fig. 4), and (iii)

identification of genetic diversity enabling estimation of penguin abundances consumed (Fig. 5).

Comparing seabird detections using diagnostic hard part and genetic analyses

Seabirds were detected in 29.3% (n = 29) of samples using diagnostic hard-parts, and 21.2% (n = 21) of samples using DNA (Fig. 3a). The majority (> 99%) of DNA sequences for each seabird taxon were identified in just 9% (n = 9) of those samples (Fig. 3a) using both a conventional and stringent standard of quality filtering and cleaning protocols (Appendix S1.3). The other 12 samples contained low amounts (< 1%) of DNA calculated relative to the total abundance of DNA obtained for each taxon (Table S5). Seabirds were detected by both methods simultaneously in only 10% (n = 10) of samples (Fig. 3a), and 5% (n = 5) samples contained both little penguin hard-parts and DNA (Fig. 3b). The other 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). While the combined proportion of samples containing either diagnostic hard-parts or DNA from seabirds, or both, amounted to 40% (n = 40) (Figs. S1a), these

dietary analysis methods represent two quasi-independent assays and we argue, are therefore not additive.

Seabird diversity in long-nosed fur seal diets

The DNA-based metabarcoding technique was more sensitive in detecting mixtures of taxa in scat samples compared to hard-part analysis (Fig. 4), with 2 distinct prey taxa detected in 5 samples and a single prey taxon in the remaining 16 samples (Fig. 4b). In contrast, diagnostic prey hard-parts typically corresponded to a single prey species within samples and no samples contained more than one identified bird taxon using this method (Fig. 4a).

Little penguins (*Eudyptula minor*) were the main seabird prey species detected using both morphological (Fig. 4a) and DNA-based analyses (Fig. 4b), both in terms of frequency of occurrence (Fig. 4b), in total abundance of sequences (Fig. S2), and in relative abundance of sequences (Fig. S3). 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). Whilst, the majority (> 99%) of little penguin DNA was obtained from 6% of samples (n = 6) (Fig. 3b & S2).

271 This study identified three other distinct seabird taxa using both dietary analysis
272 methods. Morphological analysis revealed two additional taxa: shearwaters at family level
273 (Procellaridae spp.) (n = 2 samples), and the Australasian gannet (*Morus serrator*) (n = 1)
274 (Fig. 4). DNA metabarcoding detected two distinct families of shearwater taxa in 5% (n = 5)
275 and 9.1% (n = 9) of samples, respectively (Tables S4 & S5, Appendix S2). We also identified
276 the black-browed albatross (*Thalassarche melanophris*) and greater crested tern (*Sterna*
277 *bergii*), in 1 sample apiece (Fig. 4, Table S5). The combined use of both DNA metabarcoding
278 and hard-part analysis revealed a greater diversity of taxa than would have been identified by
279 either method alone.
280
281 *Occurrence of seabird prey across southeastern Australia*
282
283 Seabirds were detected at all main sampling locations and time points, regardless of the
284 predator samples' coming from a range edge or more geographically central fur seal colony
285 (Fig. S4 & S5). Mean detection rates were statistically similar for hard parts compared to
286 DNA methods for both seabirds in general (GLM seabird detection ~ metric: p-value =
287 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 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, however these sequences did not pass DNA quality filtering procedures. Thus conservatively, we would report that whilst penguins were detected from morphological remains 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

A total of 7 little penguin mtDNA haplotypes were identified in long-nosed fur seal diets (Fig. 5a), based on selection of the most abundant unique sequences of penguin DNA within samples. All 10 samples containing little penguin DNA were subsequently searched for the presence of these 7 mtDNA haplotypes. Thus whilst 2 haplotypes were detected as being from Barunguba samples, we do detect additional haplotypes when searching those same samples for haplotypes that could come from elsewhere (Figs. 5b). Of these 10 samples, 5 contained a single haplotype, whilst 5 contained between 2–6 individual mtDNA haplotypes or individual penguins (Fig. 5b). Logically, two distinct genetic haplotypes present within a sample, represent at least two distinct individual birds consumed. Thus, we posit at least 21 individual penguins were consumed across all samples, from two sampling locations and multiple seasons.

Discussion

We leveraged recent advances in cost-effective genetic assaying tools combined with traditional diet analysis methods. We contributed the following significant advances both within our local context and to the broader conservation biology community: (i) a multi-assay method for comparison of target species identification – producing a more reliable 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, we provide conservation practitioners with a predation prevalence range for seabirds (9–29%) and little penguins (6–25%) in the diets of long-nosed fur seals in southeastern Australia. We confirm that little penguins are currently the most commonly consumed seabird by long-nosed fur seals in comparison to other seabirds (e.g., procellarids, black-browed albatross, greater crested tern, and Australasian gannet). Whilst previous

studies have identified little penguin remains at relatively low frequencies overall (5.9% of samples in Page et al. 2005, <2% in Hardy et al. 2017, ~13% of samples in Goldsworthy et al. 2019), the upper range of estimates observed in this study (25% of samples) signals a need for increased monitoring. Specifically, longer-term and comprehensive sampling programs are needed to further quantify and update the spatiotemporal patterns in consumption by long-nosed fur seals. 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 over time, with changing predator demography through population recovery and through climate change. Further, it may be that a learned behaviour becomes advantageous to a sub-population and is transmitted to other predator populations, particularly in response to environmental changes and prey availability. Analysis of the predator's total diet consumed is also warranted to gauge the relative importance of different prey items, in addition to or in combination with focusing on a specific taxonomic group such as seabirds.

Quantifying predation can be difficult for certain taxa and current DNA-based tools already offer significant advantages over identifications of morphological prey remains. 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 tear their skin and feathers off (Hocking et al., 2016). Hard-part analysis typically assigns one individual to remains such

as a pair of fish otoliths, a bird skull, paired feet or paired upper and lower cephalopod beaks, however assigning the number of individuals to remains such as feathers or fur has been simplistic (Page et al., 2005). A recent controlled feeding trial identified that the morphological remains of a single penguin could appear in up to 5 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 in an overestimation of those taxa in diet analyses and overestimation of their consumption.

Based on haplotype polymorphism, we propose that at least 21 individual penguins were consumed and occurred in only 10% of long-nosed fur seal scat samples. Further, a single scat could contain up to 6 haplotypes or individual penguins. We posit that this number is likely an underestimate, firstly due to highly 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). Secondly, this study uses 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 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 (Taberlet et al. 2012). If longer fragments are targeted, DNA traces from birds that are more digested may be lost. However, ongoing improvements in DNA extraction and sequencing techniques will ensure genetic tools remain at the forefront of wildlife forensics and ecological monitoring.

This paper ultimately posits that DNA-based methods will significantly advance wildlife conflict surveillance and impact assessment between conservation priority species. DNA metabarcoding provided key additional information here, critical to assessing predator-prey interactions within a wildlife conflict and conservation management context: (i) offering multiple metrics in addition to occurrence rates; (ii) detecting multiple prey taxa within a single sample; and (iii) identifying genetic diversity enabling estimation of penguin abundances consumed. We recommend the development and optimization of cost-effective assays tailored to the needs of specific wildlife conflict scenarios in order to better quantify and monitor these interactions. The use of multiple target genes typically produces more

392 reliable results with which to form consensus on predation prevalence and likely impacts.
393 Genetic screening for predator DNA enables individual predator identification (Wegge et al.,
394 2012) and this may be of especial interest to managers when controversial strategies are on
395 the table for controlling predation. If consumed biomass information is needed, we
396 recommend developing DNA-to-tissue-based correction factors (Thomas et al., 2014).
397 Numerous studies have developed species-specific and cost-effective assays using older
398 technology and that could be applied to large sample sizes and large numbers of predatory
399 taxa, for the detection of specific taxonomic groups of high conservation or commercial
400 interest (Fox et al., 2012; Hunter et al., 2012; Schreier et al., 2016). Skaala et al. (2014) used
401 genetic techniques not only to identify the prey species of interest, but also used several
402 microsatellite markers to identify the origin of prey stock at high spatial resolution.
403 Predator impacts need to be considered and managed within an up-to-date cumulative
404 impact assessment for threats, here to little penguins in southern Australia, before money is
405 spent on strategies that may not be effective, such as native predator culling. Like many other
406 wildlife conflict situations, endemic predation is natural, and often habitat degradation,
407 environmental change and invasive species are more significant sources of impact to
408 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 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). 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 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 figures will be made available via an online data publication repository upon acceptance of this manuscript for publication.

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632 **Figure Legends**

633

634 **Figure 1.** a) Long-nosed fur seal scat collection sites (n = total sampling effort numbered).
635 Pup abundance, as an index of seal population, has been included for sampling locations, to
636 illustrate the relative importance of these sites for long-nosed fur seal populations in
637 southeastern Australia. Sampled sites were: Cape Bridgewater (38.3013˚ S, 141.4062˚ E) and
638 nearby Deen Maar Island (formerly Lady Julia Percy Island, 38.4161˚ S, 142.0038˚ E) from
639 western Bass Strait, Victoria; Gabo Island in eastern Bass Strait, Victoria (37.5649˚ S,
640 149.9133˚ E); and Barunguba (formerly known as Montague Island 36.2510˚ S, 150.2270˚ E)
641 at the northeastern breeding range in New South Wales (NSW). Range of both species shown
642 for b) long-nosed fur seals and c) little penguins using Atlas of Living Australia distribution
643 data (ALA, 2019).

644

645 **Figure 2.** Contextual images of a) the long-nosed fur seal, *Arctocephalus forsteri*, from
646 Barunguba, NSW; b) the little penguin, *Eudyptula minor*, often burrowing near fur seal
647 colonies; and examples of seabird remains in c) and d), often found as regurgitates, from
648 long-nosed fur seal haul-outs and colonies.

649

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

656

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

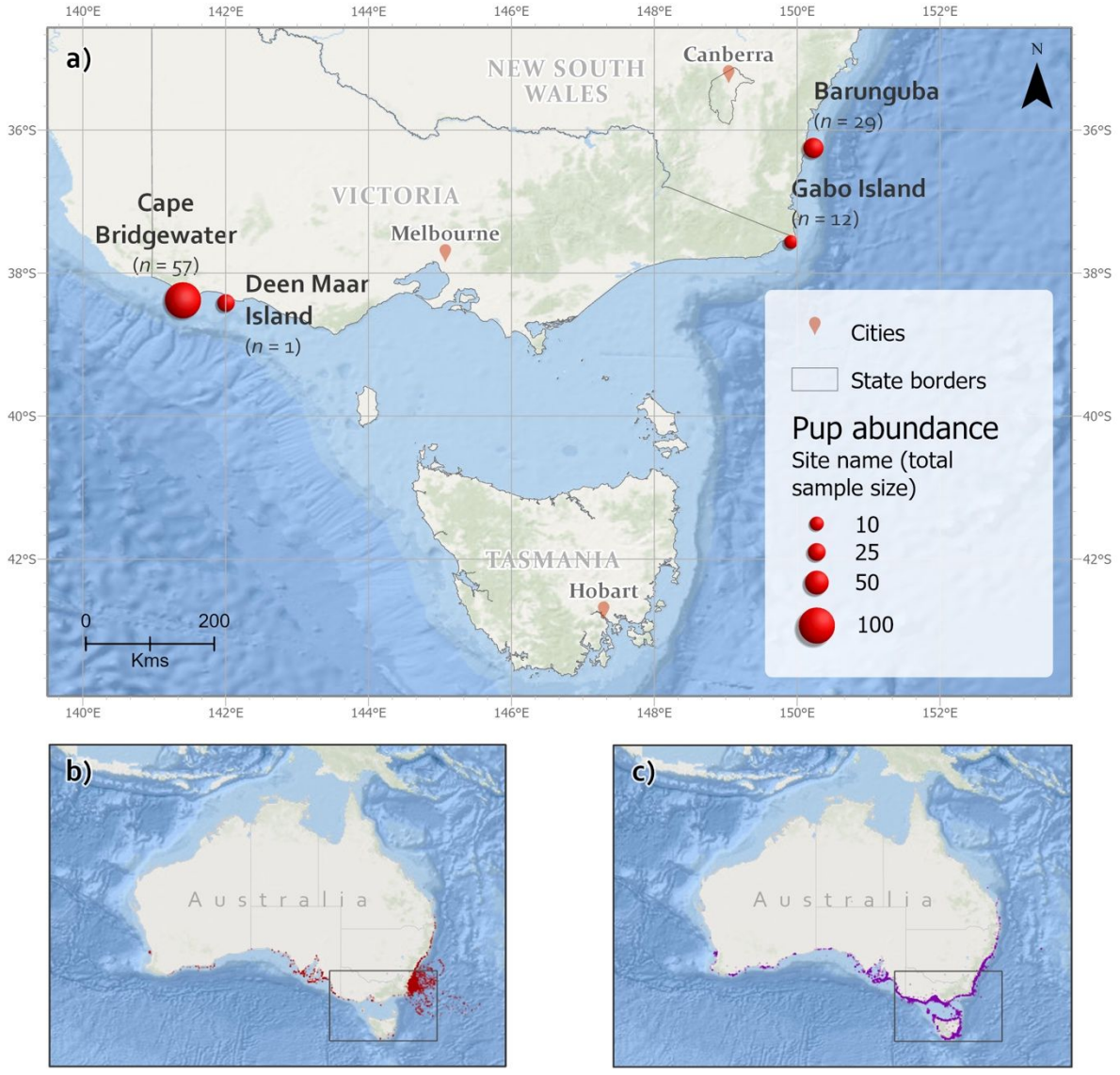
661

662 **Figure 5.** Little penguin genetic diversity (for 230 bp 12S rRNA gene) a) presented as a
663 minimum spanning network of 7 distinct haplotypes, and b) estimated number of individuals
664 consumed across the sample region and time period based on haplotype consumption,
665 including haplotype sequence abundances within samples. Numbers in each circle represent a

666 unique haplotype identifier. Here, each unique haplotype within an individual fur seal scat
667 sample represents an individual penguin consumed (b) and we overlay the genetic sequence
668 abundance identified within samples that tested positive for penguin (n = 10) for each
669 haplotype.

670 **Figures & Tables**

671



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

672

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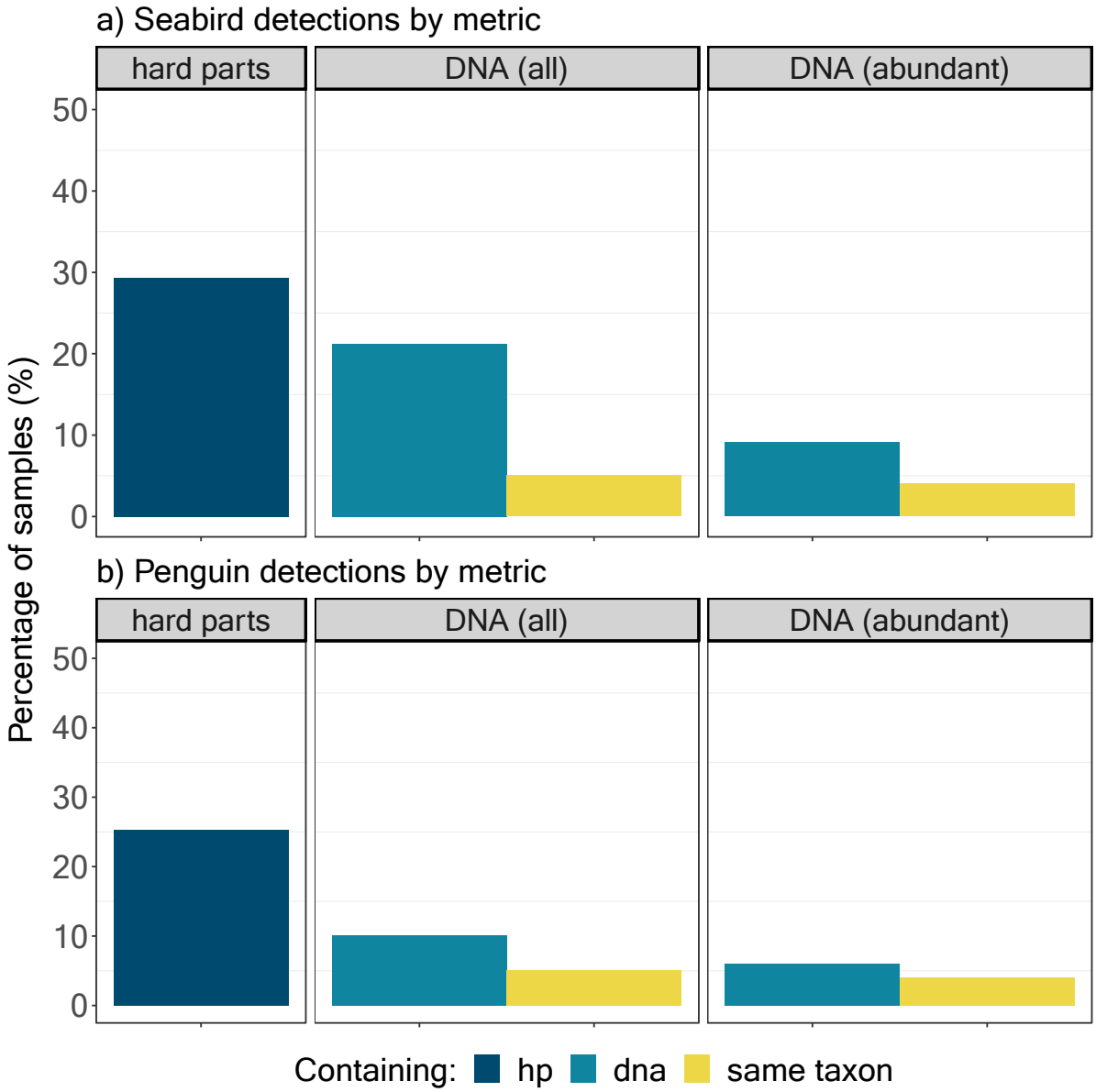

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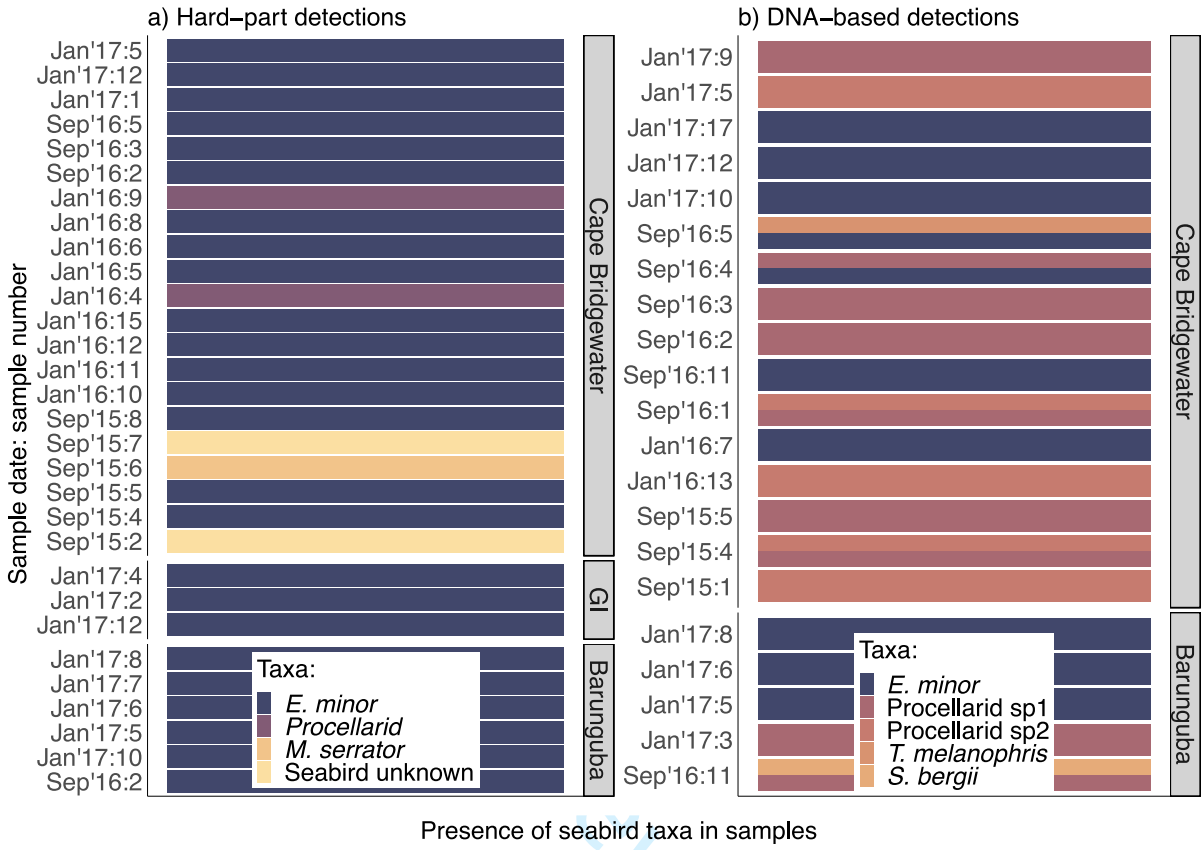

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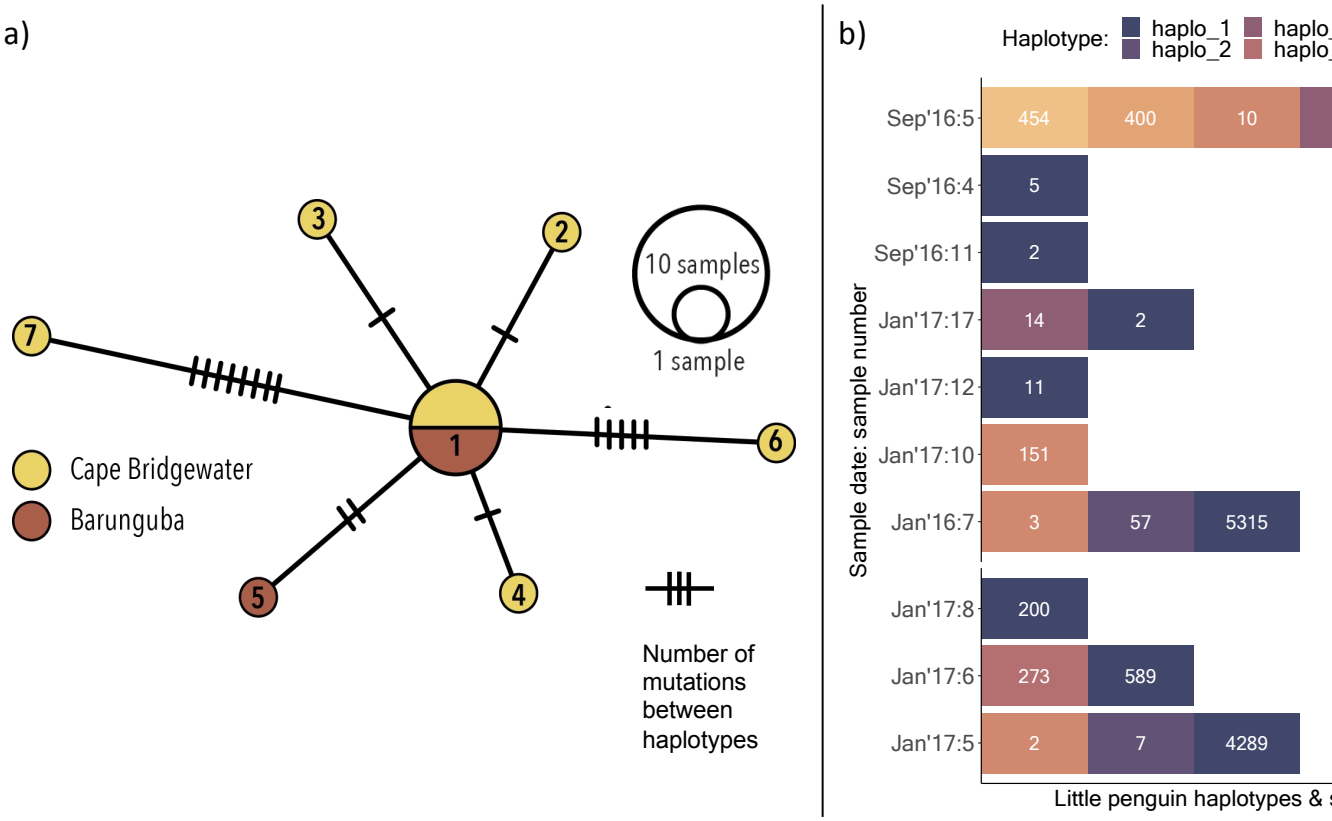

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