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DNA metabarcoding complements but does not replace direct observations of penguin predation by corvids

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Establishing methods that allow for more focused management of wildlife under predator pressure may increase the efficiency of managing problematic predators. Non-invasive dietary analysis and identification of conservation-sensitive prey in the diet of 'culprit' predator individuals could help to facilitate this and is worthy of exploration. Recently on Phillip Island, Australia, Little Ravens *Corvus mellori* have emerged as a prominent predator on the clutches of burrow-nesting Little Penguins *Eudyptula minor*. We tested the feasibility of using non-invasive PCR approaches targeting the penguin mitochondrial 16S rRNA marker gene to establish whether penguin DNA could be detected in raven faecal samples, potentially enabling the identification of culprit ravens missed by extensive field observation. Using a metabarcoding approach, we examined the feasibility of non-invasively establishing other dietary items via high-throughput amplicon sequencing. We documented components of raven diet using the universal mitochondrial 16S rRNA, insect-specific 'Chiar' 16S rRNA and plant ITS2. The assemblage of dietary items did not differ with raven culprit status (i.e. a raven previously observed preying upon penguin), sex or date. Penguin was detected in the diet of some individuals classified observationally as non-culprits. Although some cases may conceivably have been false detections, other explanations include missed depredation events, consumption via scavenging or through secondary consumption (e.g. eating invertebrates that have consumed penguin). While this study found metabarcoding unreliable for unambiguous assigning of raven culprit status, at least as we implemented it, it may hold promise complementing observations if consumption via scavenging can be distinguished from direct depredation.

Keywords: hunting, Little Penguin, predators, prey, scavenging, Little Raven.

The adaptability of species with generalist diets allows access to a wide array of food available in an environment. In the case of biological invaders, the capacity to forage on a variety of food can facilitate their success in adapting to novel settings and exploiting new resources (Sol *et al.* 2011, Magory Cohen *et al.* 2020, Tan *et al.* 2021). Within populations, generalist foragers may still develop foraging specialisations (e.g. Dickman & Newsome 2015), which can be driven by morphological differences between individuals of a species (Durell *et al.* 1993, Cook *et al.* 2013) or by the development of innovative behaviour to take advantage of new high-value food sources (Hunt 2000, Ekanayake *et al.* 2015a). Such

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specialisation can take the form of focused predatory activities on specific prey (Durell *et al.* 1993, Hunt 2000). Under circumstances in which the prey species is ecologically sensitive (i.e. demographically vulnerable to high mortality rates), evolutionarily naïve and/or poorly adapted to deal with the new predatory threat, detrimental consequences to the prey species' survival can occur (Schoener *et al.* 2001, Doherty *et al.* 2016). Under such circumstances, management intervention may be desirable.

Pest predator management efforts attempt to mitigate the impact on vulnerable prey, and for non-native pests, these methods often include culling or removal of the offending species (Regehr *et al.* 2007, Kirkwood *et al.* 2014). However, native biological invaders also exist (Ueta *et al.* 2003, Ueta & Hirano 2006, Carey *et al.* 2012) and may require management intervention. In some cases, complete pest eradication can be achieved (Phillip Island Nature Parks (PINP) Annual Report 2018–2019); however, even partial pest predator removals can still result in positive impacts on prey (Bolton *et al.* 2007). In other cases, removed predators might be quickly replaced by others in the population (Bolton *et al.* 2007) and, as such, removal may not present a sustainable long-term management solution. Identifying whether certain individuals are more problematic than others (i.e. identifying 'culprits') might assist in targeted control methods, thereby informing the effectiveness of management decisions (Tan *et al.* 2021).

Dietary and foraging specialisations of wild animals have typically been identified by observational methods (e.g. Durell *et al.* 1993, Hunt 1996, Ekanayake *et al.* 2015a) and morphological identification (i.e. of undigested, physical items) of faecal, gut or stomach contents (e.g. Green 1966, Rowley & Vestjens 1973). More recently, molecular methods have revealed dietary specialisations and seasonal foraging habits in various species (Deagle *et al.* 2010, Ando *et al.* 2016, Gable *et al.* 2018, Voelker *et al.* 2020). Dietary analysis facilitates our understanding of predator–prey interactions, possibly identifying specific individuals or groups that are specialised on certain prey types and potentially driving population declines. These methods can elucidate food web structures and foraging ecology (Hobson 1995, Deagle *et al.* 2010, Cavallo *et al.* 2018, Gable *et al.* 2018, Toju & Baba 2018, Shutt *et al.* 2020), as well as dietary shifts in response to changes in the environment or in food availability (Hobson 1995, Hobson *et al.* 1999, Gable *et al.* 2018, McClenaghan *et al.* 2019).

Faecal analysis can reveal dietary specialisations and conservation-relevant predator–prey interactions (Oja *et al.* 2017, Voelker *et al.* 2020). Molecular analyses have greatly broadened our knowledge of diet from DNA extracted from gut, faecal and stomach contents (de Sousa *et al.* 2019). Molecular analyses of faecal matter (faecal metabarcoding) have provided a non-invasive way of accurately establishing specific prey items of organisms in several systems (Gerwing *et al.* 2016, Oja *et al.* 2017, Cavallo *et al.* 2018, Gable *et al.* 2018, de Sousa *et al.* 2019, Thuo *et al.* 2019, Shutt *et al.* 2020). Specifically, metabarcoding may detect some items that would otherwise be missed using only traditional methods (de Sousa *et al.* 2019). Information on diet derived from faecal samples is highly linked to digestion time, reflecting a relatively current 'snapshot' of diet spanning the period from consumption to faecal deposition (Culliney *et al.* 2012, Simonová *et al.* 2016, Thuo *et al.* 2019). Thus, faecal metabarcoding can be used as a tool to establish dietary plasticity across time (Gable *et al.* 2018, McClenaghan *et al.* 2019, Shutt *et al.* 2020, Voelker *et al.* 2020) and so holds promise to monitor any temporal shifts in dietary composition.

Many corvids are generalist omnivorous species that have the potential to develop specific foraging niches, or at least heavily exploit certain prey species within an environment (Hunt 2000, Ekanayake *et al.* 2015a). In Australia, frequent depredation of Little Penguin *Eudyptula minor* clutches by native Little Ravens *Corvus mellori* has been identified as an emergent conservation issue on Phillip Island, Victoria (Nakazawa 2003, Swinburne & Jessop 2005, Ekanayake *et al.* 2015a). Following decades of extensive management efforts (including fox eradication and a land buy-back scheme; PINP Annual Report 2010–2011, Kirkwood *et al.* 2014), Phillip Island now boasts an estimated population of *c.* 31 000 breeding adult Little Penguins (Sutherland & Dann 2014) that are also the focus of an ecotourism attraction (PINP Annual Report 2018–2019). Due to the intense nature of raven depredation on penguin clutches (Ekanayake *et al.* 2015a, Tan *et al.* 2021), this ecologically and economically important penguin colony is considered under some level of risk and mitigative solutions to this depredation have been sought.

Burrow-raiding ravens work solely or in pairs, gaining access to clutches directly via the burrow

entrance or by excavating an additional hole in the burrow chamber, also bypassing a defending adult penguin (Ekanayake *et al.* 2015a). With some exceptions, corvids are typically abundant and exhibit highly flexible foraging strategies (Barrett *et al.* 2003, Brook *et al.* 2003, Ryall 2016). Peak Little Raven abundance on Phillip Island's Summerland Peninsula coincides with peak Little Penguin breeding (Ekanayake *et al.* 2015b). Their high abundance locally (in absolute terms) and propensity to move considerable distances suggests any removed ravens are likely to be quickly replaced by others in the population (Barrett *et al.* 2003, Bolton *et al.* 2007, Whisson *et al.* 2015). Thus, efforts have been made to establish whether 'culprit' (burrow-raiding) behaviour was restricted only to certain individuals which could be selectively managed. However, culprits were not more genetically related to each other than non-culprits, suggesting burrow-raiding is not a behaviour learnt within and limited to certain family groups (Tan *et al.* 2021). Similarly, although some morphological differences exist between culprit and non-culprit ravens, they are subtle and insufficient to assign culprit status confidently (Tan *et al.* 2022). Thus, any such differentiation or identification of birds as culprits, should such specialisations exist, remains elusive.

Most of the information available on Australian corvid diets stems from research conducted 50 years ago (Rowley & Vestjens 1973), primarily within rural, farmland and non-coastal areas (Rowley 1973). Information on the diet of Australian corvids is currently restricted to stomach contents analysis and observations (e.g. Green 1966, Rowley & Vestjens 1973, Swinburne & Jessop 2005, Lill & Hales 2015, Clifton & Jones 2017, Sazima 2020). Comprehensive and recent information on corvid diet is lacking and modern dietary analysis tools have not yet been attempted or applied. Here we assess the use of faecal metabarcoding and whether it can be used to supplement the identification of culprits that may have been missed by observational methods. In doing so, we also document the diet of a population of Little Ravens living in a coastal and rare fox-free habitat in southeastern Australia.

For terrestrial birds, applications of faecal metabarcoding include being used to examine the availability of prey vs. actual prey consumed in four species (Rytkönen *et al.* 2019), to reveal dietary flexibility of another species (McClenaghan *et al.* 2019), to establish dietary richness and detect seasonal dietary shifts that may occur (Shutt *et al.* 2020) and even to place economic value on the service of birds as controllers of pest populations (Garfinkel *et al.* 2020). However, the use of DNA metabarcoding in avian dietary studies is still relatively new. As reviewed by de Sousa *et al.* (2019), birds account for *c.* 17.7% of the 277 taxa examined across 165 studies employing metabarcoding and, to our knowledge, dietary metabarcoding has yet to be applied to any corvid species (but see Juozaitytė-Ngugu *et al.* 2021) who use molecular methods to detect parasites in intestinal samples of corvids in Lithuania).

The two aims of this study are: (1) to examine whether metabarcoding techniques allow for the detection of Little Penguin DNA in raven faecal samples, and determine whether this corresponds with known culprits of penguin depredation detected by observational methods; and (2) to contextualise consumption of penguin in the overall raven diet. By establishing the identity of known culprits, in conjunction with other information on how problematic new behaviours spread through a population, more focused long-term management efforts to mitigate penguin predation may conceivably be applied.

METHODS

Data collection

Raven trapping and establishing culprit status of individual birds

Phillip Island's primary penguin colony is located on the Summerland Peninsula (south-central Victoria, Australia; 146°22′E, 38°04′S). In total, 198 ravens were caught and banded from September 2012 to October 2013, and May 2015 to February 2017 across the peninsula. A further two birds were trapped in 2018 to obtain known positive control samples (see below). The primary method of trapping involved baited standard cat traps and a modified Australian Crow Trap (see Tan *et al.* 2021). Bait consisted of bread and dog food, with dog food ingredients listed as containing meat (chicken, lamb, beef, pork) and vegetable fibre. We selected effective bait for capture but acknowledge that for omnivores which exploit a vast array of natural and supplemented food, there was inevitably a risk that detection of our bait in faeces may mask consumption of similar prey consumed by real birds. Ravens were typically in traps

no longer than 3–4 h. Ravens were occasionally caught as by-catch as part of Phillip Island Nature Park's cat-trapping programme, where rabbit and shearwater were used as bait (faecal samples were not collected from these traps). All ravens were banded and colour-marked for identification, and later sexed genetically from blood and/or feather samples (Tan *et al.* 2021).

We established individual identity of burrow-raiding 'culprit' ravens using images captured by remote-sensor cameras placed at penguin burrows across three penguin breeding seasons (2013–2014, 2015–2016 and 2016–2017; 6.4 million images; Tan *et al.* 2021) supplemented by direct observations (660 field days). From these, three groups were established: 'Definite Culprits', 'Possible Culprits' and 'Other Birds'. Definite Culprits were banded individuals that had been identified actively preying on penguin eggs or small chicks at burrows (6 females; 8 males). Possible Culprits were banded individuals that were seen visiting penguin burrows but with no evidence of active depredation (18 females; 31 males). All remaining banded ravens were classified as 'Other Birds', i.e. individuals that had been banded but not seen at penguin burrows (63 females; 72 males). We note that, for 'Other Birds', we cannot be entirely certain that these birds are truly non-culprits.

Faecal sample collection

Raven faecal samples were collected during the 2016/2017 penguin breeding season via opportunities during banding (i.e. faecal material deposited by ravens while being handled), and from cardboard placed at the base of cat traps that captured ravens. Cardboard was replaced each time a trap was successfully triggered by an animal to prevent contamination. Typically, only one raven was caught in a cat trap at a time and faecal samples could be assigned to that individual. We excluded samples from the rare occasions when more than one individual was found in a cat trap or faecal samples could not be confidently assigned to the individual in the trap. Samples were scraped directly into a collection tube of 90% ethanol using a toothpick or the edge of the collection tube and placed in refrigerated storage (usually within 24 h), and freezer storage at the end of the field season once transported to the laboratory. In April 2018, we obtained definite positive control samples from a local raven that was confirmed to have ingested penguin tissue (Supplementary online material (SOM) Table S1, SOM Appendix S1).

Genetic analyses

Whole genomic DNA was extracted from faecal samples using Qiagen DNeasy extraction kits (Qiagen Inc., Valencia, CA, USA) following the manufacturer's protocol for extraction from tissue samples. A variety of polymerase chain reaction (PCR) primers were applied to address each aim (Fig. 1). In this study we examine results from mitochondrial 16S rRNA using universal animal primers (Sarri *et al.* 2014) and primers more specific to insects (Chiar 16S; Marquina *et al.* 2019); the plant-specific ITS2 (Cheng *et al.* 2016); and a custom primer developed for this study designed to be penguin-specific (Table S1). The barcoding locus COI (Leray *et al.* 2013) was also used; however, we excluded these data due to contamination from an unrelated project. A custom blocking primer was used to suppress PCR-amplification of corvid DNA and enhance the detection of prey DNA (henceforth 'BLOCK'; Table S1; Vestheim & Jarman 2008); however, it was unsuccessful and hence was not applied to the datasets examined here. The primers used to PCR-amplify regions of DNA in this study were run together in a single multiplex reaction (Fig. 1).

Initial feasibility trial

We conducted an initial feasibility trial (Fig. 1) using mitochondrial 16S rRNA on a 'test' dataset; i.e. eight faecal samples from raven individuals that were suspected of most probably having consumed penguin (Definite and Possible Culprits established from camera footage at the time, before all 6.4 million images had been reviewed). Next-generation sequencing (NGS) on these samples was performed by Monash University Malaysia Genomics Facility. Initial results did not detect Little Penguin in any samples; however, they provided an overview of other potential dietary items and confirmed that the remaining raven faecal samples could be successfully examined with mitochondrial 16S rRNA (see below).

Positive control samples

Following the feasibility trial, positive control samples were obtained (from a captive raven which was fed penguin; see above) and we confirmed that penguin DNA could be detected in gel reads

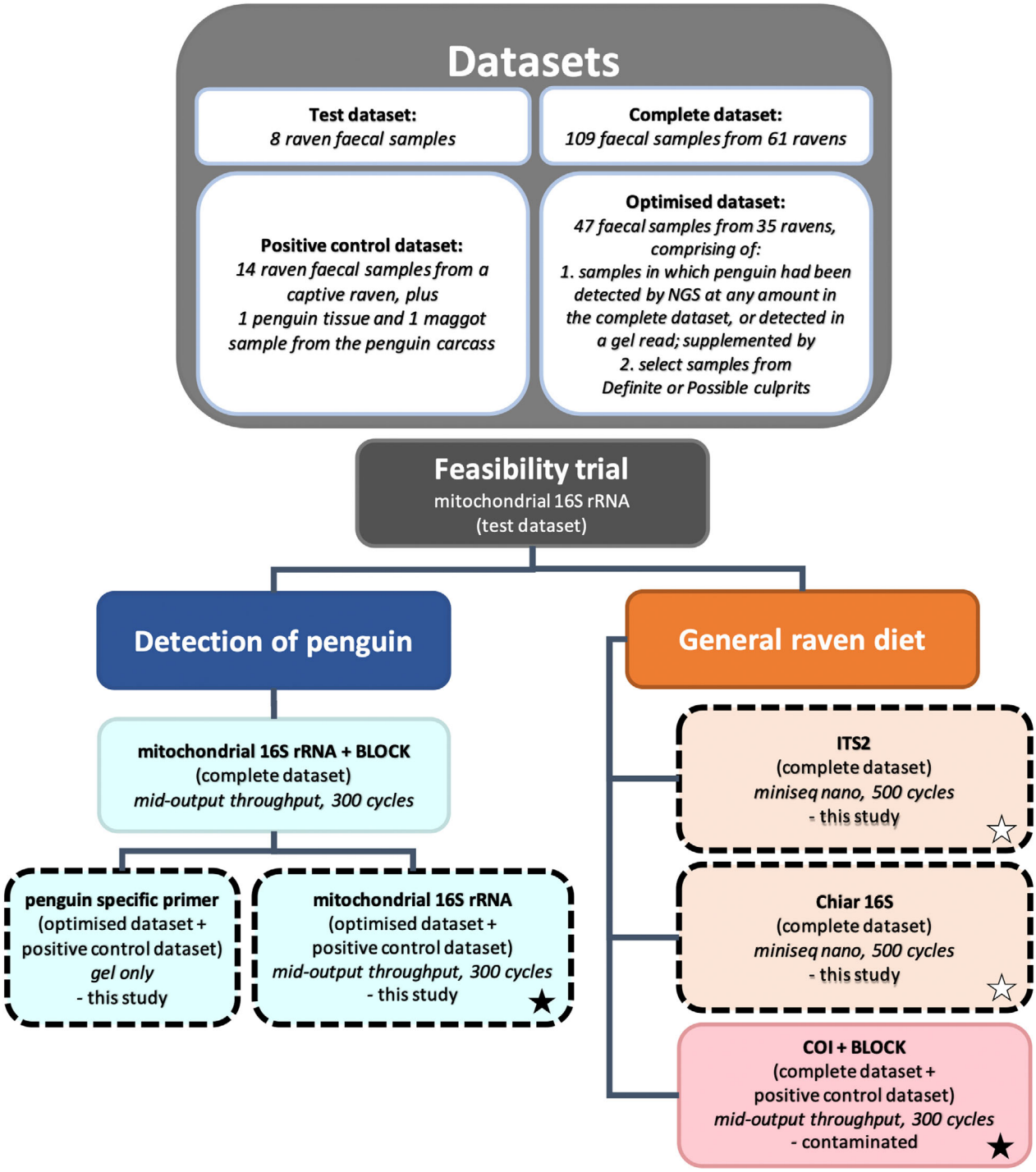

Figure 1. Datasets used in this study and flow chart demonstrating the process to establish: (1) detection of penguin and (2) general raven diet. Star symbols represent datasets and primers multiplexed together (by black stars and white stars). A 'test' dataset was used to test the feasibility of using raven faecal samples with DNA-based dietary detection methods, and a 'positive control' dataset was used to confirm detection of penguin in raven faecal samples. A 'complete' dataset was used comprising all available faecal samples (separate from positive controls) to determine general raven diet and contextualise the role of penguin in that diet. To enhance detection of penguin, we used an 'optimised' dataset focusing on culprit birds and samples with positive reads for penguin. Processes outlined with a dotted border represent those used in this study.

using the custom penguin-specific primer (see 'Protocols' below). This 'positive control' dataset contained 14 samples from the positive control bird, plus two positive controls of: (1) penguin tissue and (2) maggots sampled from inside the penguin carcass which was consumed by the positive control bird and which were found to contain penguin in empirical trials. Hence, we established that maggot samples could also be used for the detection of penguin DNA.

Aim 1: Can Little Penguin be detected in the diet of ravens?

Following the feasibility trial, two datasets were used to screen for penguin DNA in the diet of ravens and therefore examine any correspondence with known culprits (Fig. 1). One was a 'complete' dataset; this consisted of 109 samples from 61 individual ravens collected during 2016–2017. The other was an 'optimised' dataset (47 samples from 35 individuals) comprising samples in which penguin DNA had been detected by NGS at any amount in the complete dataset (see 'Protocols' below) or were detected in a gel read; these were supplemented with a selection of samples already identified as Definite or Possible culprits (see Fig. 1 and below). The purpose of the optimised dataset was to maximise detection of penguin DNA using NGS. In this study, we examine the results of the optimised dataset plus the positive control dataset against mitochondrial 16S rRNA and the penguin-specific PCR primer.

Aim 2: Examining overall raven diet in relation to the consumption of penguin

To determine general raven diet and contextualise the role of penguin in that diet, we used all available samples (i.e. the 'complete' dataset) against different primers (Fig. 1). Here we examine the results of the complete dataset against Chiar 16S and ITS2.

Protocols

PCR was performed in 10 μL volumes, inclusive of 0.2 μL of each primer. For initial samples (trial with mitochondrial 16S rRNA, mitochondrial 16S rRNA + BLOCK with all samples, and penguin-specific primer) a 5-μL mastermix (2 μL buffer, 1 μL $MgCl_2$, 1 μL dNTP, 0.1 μL polymerase, 0.9 μL H_2O); 3.6 μL H_2O; and 1 μL gDNA was used, plus primer. For later samples (mitochondrial 16S rRNA, Chiar 16S, ITS2, COI + BLOCK) we used 5 μL Q5® Hot Start High-Fidelity 2X Master Mix (New England Biolabs), which contains Q5 Hot Start High-Fidelity DNA Polymerase; 3.6 μL H_2O; and 1 μL gDNA, plus primer. PCR thermal cycling conditions were 94 °C for 5 min; then 40 cycles with 94 °C for 30 s, 50 °C for 30 s and 72 °C for 15 s. A final extension occurred at 72 °C for 1 min following the last cycle. For samples with Q5 Hot Start High-Fidelity DNA Polymerase, thermal cycling conditions were 98 °C for 30 s; then 35 cycles with 98 °C for 5 s, 55 °C for 20 s and 72 °C for 20 s. A final extension occurred at 72 °C for 2 min following the last cycle. Where applicable (i.e. to visualise the penguin-specific primer data, and test PCR success for samples run against mitochondrial 16S rRNA and Chiar 16S), PCR products were separated on a 1% agarose gel and run in standard sodium borate buffer, visualised using GelRed™ (Biotium, Inc.) nucleic acid gel stain.

Samples were purified using Omega Biotek Mag-Bind Total Pure NGS (Custom Science, Victoria, Australia) to discard unnecessary fragments, and then barcoded before pooling. NGS on the complete dataset was conducted for mitochondrial 16S rRNA (with BLOCK) using an Illumina MiniSeq mid-output kit (300 cycles), and Chiar 16S and ITS2 using an Illumina MiSeq reagent nano kit (500 cycles). Similarly, the optimised plus positive control datasets for mitochondrial 16S rRNA were examined using an Illumina MiniSeq mid-output kit (300 cycles). Samples were run with primers as per Figure 1 and Deakin University in-house genomic services were used for all runs. The use of BLOCK was unsuccessful in suppressing raven DNA PCR-amplification, both from empirical tests and from NGS against all samples with mitochondrial 16S rRNA. We aimed to re-test it using COI but the batch of data was heavily contaminated with an unrelated species and could not be analysed.

Bioinformatic analyses

Sequencing reads were trimmed of their PCR primer sequences followed by dereplication, error-correction (denoising) and clustering at 97% similarity using USEARCH v11 (Edgar 2010). To generate an operational taxonomic unit (OTU) count table, the trimmed reads were aligned back to the OTUs using the 'Usearch_global' command. Taxonomic assignment of the OTUs used the 'sintax'

command with a confidence score cut-off of 0.8 based on the MIDORI reference database (Leray *et al.* 2018, Banchi *et al.* 2020). The OTU table, taxonomic assignment data and sample metadata were submitted to MicrobiomeAnalyst for data visualisation (Dhariwal *et al.* 2017). We calculated presence/absence (and the percentage composition; see SOM Appendix S2) of each OTU from count data (number of reads per OTU), after removal of OTUs containing corvid host data and human DNA contamination from the 16S rRNA and Chiar 16S datasets. Low-level reads for the OTU comprising members of the genus *Chenopodium* and species *Tetragonia implexicoma* were detected in one of the two H_2O control samples from the ITS2 dataset (read count of 3 and 2, respectively). We opted to retain these OTUs in analysis due to the low proportion detected in the control compared with other samples (i.e. average read count of 281.65 and 816.14 for *Chenopodium* and *Tetragonia implexicoma*, respectively, $n = 99$) and because not all samples in the run detected these OTUs (suggesting contamination did not occur across all samples). Analyses conducted excluding *Chenopodium* and *Tetragonia implexicoma* (Appendix S2) revealed no qualitative difference in outcomes (see Results).

Using presence/absence data of each OTU, we examined datasets by: (1) Class (16S rRNA and Chiar 16S) and Subclass (ITS2) level (excluding any OTU data that were not at least Class/Subclass-specific, and summing remaining OTU data by Class/Subclass); and (2) Species level (excluding any OTU data not at least Order-specific). We examined Subclass for ITS2 data as all but one OTU fell within a single Class (Table 1) and Subclass was the next level of resolution available. The Species-level dataset included OTUs with information for at least Order-level specificity, as most identifications within this analysis were to the Species level except in a few cases ($n = 12$; Table 2). We acknowledge that a Species-level match in the OTU database only indicates that the reference sample was identified to Species level (and that reads may not be the same exact species) and have interpreted that information accordingly. For example, the Large-billed Crow *Corvus macrorhynchus* was detected by NGS in this study, despite not occurring in Australia; but was interpreted in this study as being Little Raven host DNA. Similarly, the Order, Family or Genus information could still distinguish those OTUs from other species detected. For example, OTU 18 in Table 2 provides only Genus-level information but is unique and, in conjunction with existing knowledge of taxa on Phillip Island, this OTU may tentatively be assigned to Short-tailed Shearwaters *Ardenna tenuirostris*, a colonial nesting seabird abundant at that location.

Statistical analyses

We acknowledge that factors including variability in digestion rate, prey biomass and PCR primer bias may affect the relative abundance of NGS reads and hence presence/absence analyses are presented in this paper (e.g. Berry *et al.* 2017). However, we present identical analyses on the compositional data in Appendix S2.

We took a conservative approach whereby any read attributed to penguin DNA constituted the presence of a penguin, as our priority was to detect any penguin consumed (reads for penguin ranged from 13 to 734 across eight samples in the optimised dataset). While we note that this approach may increase the possibility of false detections, we considered this less problematic than missing detection of penguin DNA. To examine differences in the presence/absence of OTUs detected between culprit status (Definite, Possible and Other) and sex (male, female), we used PRIMER v.7 (Anderson *et al.* 2008) to conduct two-factor permutational multivariate analyses of variance (PERMANOVA with Type 1 SS) specifying raven identity (ID) as a random effect to account for a lack of independence between samples of the same individual. Analyses were based on Jaccard zero-inflated resemblance matrices. Terms were ordered with the covariate first, followed by culprit status, sex, culprit status*sex interaction, and the random factor of ID last. We ran all six possible orders of the fixed/interaction terms to ensure results did not qualitatively differ. We found no qualitative differences and present results as per the order above. All analyses include the covariate of Julian date to account for possible temporal shifts in diet (e.g. possible seasonality in prey availability).

To address Aim 1, we conducted a PERMANOVA on the optimised dataset against mitochondrial 16S rRNA. To address Aim 2, we conducted a PERMANOVA on the complete dataset against Chiar 16S. We examine only the optimised dataset for mitochondrial 16S rRNA here (vs. the

Table 1. List of detected taxa within the complete dataset against ITS2.

OTU	Phylum	Class	Sub-class	Order	Family	Genus	Species	Common Name	% of samples detected
1	p:Streptophyta	c:Magnoliopsida	sc:Asteranae	o:Asterales	f:Asteraceae	g:*Arctotheca*		g:Capeweed	8.08
2	p:Streptophyta	c:Magnoliopsida	sc:Asteranae	o:Asterales	f:Asteraceae	g:*Helianthus*		g:Sunflower	1.01
3	p:Streptophyta	c:Magnoliopsida	sc:Asteranae	o:Asterales	f:Asteraceae	g:*Senecio*		g:Ragwort, Groundsel	2.02
4	p:Streptophyta	c:Magnoliopsida	sc:Asteranae	o:Asterales	f:Asteraceae	g:*Solenogyne*		g:Australian Daisies	1.01
5	p:Streptophyta	c:Magnoliopsida	sc:Asteranae	o:Asterales	f:Asteraceae	g:*Soliva*	s:*Soliva_sessilis*	s:Field Burrweed	1.01
6	p:Streptophyta	c:Magnoliopsida	sc:Asteranae	o:Asterales	f:Asteraceae			f:Sunflowers	12.12
7	p:Streptophyta	c:Magnoliopsida	sc:Asteranae	o:Ericales	f:Ericaceae	g:*Leucopogon*	s:*Leucopogon_parviflorus*	s:Coast Beard-heath	2.02
8	p:Streptophyta	c:Magnoliopsida	sc:Asteranae	o:Ericales	f:Ericaceae			f:Heaths	2.02
9	p:Streptophyta	c:Magnoliopsida	sc:Asteranae	o:Lamiales	f:Oleaceae	g:*Fraxinus*	s:*Fraxinus_excelsior*	s:European Ash	1.01
10	p:Streptophyta	c:Magnoliopsida	sc:Asteranae	o:Lamiales	f:Plantaginaceae	g:*Plantago*		g:Plantains, Indianwheat	4.04
11	p:Streptophyta	c:Magnoliopsida	sc:Asteranae	o:Lamiales	f:Scrophulariaceae	g:*Myoporum*		g:Figworts	6.06
12[a]	p:Streptophyta	c:Magnoliopsida	sc:Caryophyllanae	o:Caryophyllales	f:Aizoaceae	g:*Tetragonia*		g:Tetragonia, New Zealand Spinach	27.27
13	p:Streptophyta	c:Magnoliopsida	sc:Caryophyllanae	o:Caryophyllales	f:Aizoaceae	g:*Tetragonia*	s:*Tetragonia_acanthocarpa*	s:Tetragonia acanthocarpa	2.02
14[a,b]	p:Streptophyta	c:Magnoliopsida	sc:Caryophyllanae	o:Caryophyllales	f:Aizoaceae	g:*Tetragonia*	s:*Tetragonia_implexicoma*	s:Bower Spinach	91.92
15[a]	p:Streptophyta	c:Magnoliopsida	sc:Caryophyllanae	o:Caryophyllales	f:Aizoaceae			f:Fig-marigold, Ice Plants	19.19
16	p:Streptophyta	c:Magnoliopsida	sc:Caryophyllanae	o:Caryophyllales	f:Caryophyllaceae	g:*Cerastium*		s:Chickweed, Mouse-ear Chickweed	2.02
17	p:Streptophyta	c:Magnoliopsida	sc:Caryophyllanae	o:Caryophyllales	f:Caryophyllaceae	g:*Polycarpon*	s:*Polycarpon_tetraphyllum*	s:Fourleaf Manyseed	1.01
18	p:Streptophyta	c:Magnoliopsida	sc:Caryophyllanae	o:Caryophyllales	f:Caryophyllaceae	g:*Sagina*		g:Pearlwort	3.03
19	p:Streptophyta	c:Magnoliopsida	sc:Caryophyllanae	o:Caryophyllales	f:Caryophyllaceae	g:*Stellaria*		g:Starwort	17.17
20	p:Streptophyta	c:Magnoliopsida	sc:Caryophyllanae	o:Caryophyllales	f:Chenopodiaceae	g:*Atriplex*	s:*Atriplex_cinerea*	s:Grey Saltbush	4.04
21[a,b]	p:Streptophyta	c:Magnoliopsida	sc:Caryophyllanae	o:Caryophyllales	f:Chenopodiaceae	g:*Chenopodium*		g:Goosefoot	42.42
22	p:Streptophyta	c:Magnoliopsida	sc:Caryophyllanae	o:Caryophyllales	f:Chenopodiaceae	g:*Maireana*		g:Maireana	31.31
23	p:Streptophyta	c:Magnoliopsida	sc:Caryophyllanae	o:Caryophyllales	f:Chenopodiaceae			f:Goosefoot	2.02
24	p:Streptophyta	c:Magnoliopsida	sc:Caryophyllanae	o:Caryophyllales	f:Phytolaccaceae	g:*Phytolacca*		g:Pokeweed	1.01

(continued)

Table 1. (continued)

OTU	Phylum	Class	Sub-class	Order	Family	Genus	Species	Common Name	% of samples detected
25	p:Streptophyta	c:Magnoliopsida	sc:Caryophyllanae	o:Caryophyllales	f:Polygonaceae	g:*Fagopyrum*	s:*Fagopyrum_esculentum*	s:Buckwheat	3.03
26	p:Streptophyta	c:Magnoliopsida	sc:Lilianae	o:Poales	f:Poaceae	g:*Aegilops*		g:Goatgrass	26.26
27[a]	p:Streptophyta	c:Magnoliopsida	sc:Lilianae	o:Poales	f:Poaceae	g:*Bromus*		g:Bromes	44.44
28	p:Streptophyta	c:Magnoliopsida	sc:Lilianae	o:Poales	f:Poaceae	g:*Cenchrus*		g:Kikuyu Grass, Sandbur, Fountain Grass	3.03
29	p:Streptophyta	c:Magnoliopsida	sc:Lilianae	o:Poales	f:Poaceae	g:*Dactylis*	s:*Dactylis_glomerata*	s:Cocksfoot, Orchard Grass	2.02
30	p:Streptophyta	c:Magnoliopsida	sc:Lilianae	o:Poales	f:Poaceae	g:*Lolium*		g:Ryegrass	12.12
31[a]	p:Streptophyta	c:Magnoliopsida	sc:Lilianae	o:Poales	f:Poaceae	g:*Lolium*	s:*Lolium_multiflorum*	s:Annual Ryegrass, Italian Ryegrass	44.44
32	p:Streptophyta	c:Magnoliopsida	sc:Lilianae	o:Poales	f:Poaceae	g:*Panicum*	s:*Panicum_miliaceum*	s:Proso Millet, Common Millet	4.04
33[a]	p:Streptophyta	c:Magnoliopsida	sc:Lilianae	o:Poales	f:Poaceae	g:*Poa*		g:Bluegrass	27.27
34	p:Streptophyta	c:Magnoliopsida	sc:Lilianae	o:Poales	f:Poaceae	g:*Poa*	s:*Poa_infirma*	s:weak Bluegrass	9.09
35	p:Streptophyta	c:Magnoliopsida	sc:Lilianae	o:Poales	f:Poaceae	g:*Secale*		g:Rye	7.07
36	p:Streptophyta	c:Magnoliopsida	sc:Lilianae	o:Poales	f:Poaceae	g:*Sorghum*		g:Sorghum	2.02
37[c]	p:Streptophyta	c:Magnoliopsida	sc:Lilianae	o:Poales	f:Poaceae	g:*Triticum*		g:Wheat	45.45
38	p:Streptophyta	c:Magnoliopsida	sc:Lilianae	o:Poales	f:Poaceae			f:Grasses	5.05
39	p:Streptophyta	c:Magnoliopsida	sc:Proteanae	o:Proteales	f:Platanaceae	g:*Platanus*		g:Sycamore	1.01
40	p:Streptophyta	c:Magnoliopsida	sc:Proteanae	o:Proteales	f:Proteaceae	g:*Telopea*	s:*Telopea_oreades*	s:Gippsland/ Mountain/ Victorian Waratah	1.01
41	p:Streptophyta	c:Magnoliopsida	sc:Rosanae	o:Brassicales	f:Brassicaceae	g:*Brassica*		g:Mustard	3.03
42	p:Streptophyta	c:Magnoliopsida	sc:Rosanae	o:Fabales	f:Fabaceae	g:*Acacia*		g:Wattles, Acacias	2.02
43	p:Streptophyta	c:Magnoliopsida	sc:Rosanae	o:Fabales	f:Fabaceae	g:*Glycine*	s:*Glycine_max*	s:Soybean	11.11
44	p:Streptophyta	c:Magnoliopsida	sc:Rosanae	o:Malpighiales	f:Linaceae	g:*Linum*		g:Flax	3.03
45[a]	p:Streptophyta	c:Magnoliopsida	sc:Rosanae	o:Malvales	f:Malvaceae	g:*Gossypium*		g:Cotton	2.02
46	p:Streptophyta	c:Magnoliopsida	sc:Rosanae	o:Myrtales	f:Myrtaceae	g:*Eucalyptus*		g:Gums, Eucalypts	6.06
47	p:Streptophyta	c:Magnoliopsida	sc:Rosanae	o:Myrtales	f:Myrtaceae	g:*Melaleuca*	s:*Melaleuca_armillaris*	s:Bracelet Honey Myrtle	14.14
48[a]	p:Streptophyta	c:Magnoliopsida	sc:Rosanae	o:Oxalidales	f:Oxalidaceae	g:*Oxalis*		g:Wood Sorrel	8.08

(continued)

Table 1. (continued)

OTU	Phylum	Class	Sub-class	Order	Family	Genus	Species	Common Name	% of samples detected
49	p:Streptophyta	c:Magnoliopsida	sc:Rosanae	o:Rosales	f:Rosaceae	g:*Prunus*		g:Chokecherry, Plum	2.02
50	p:Streptophyta	c:Pinopsida	sc:Pinidae	o:Pinales	f:Cupressaceae	g:*Hesperocyparis*	s:*Hesperocyparis_arizonica*	s:Arizona Cypress	3.03

The percentage of samples that each taxon was detected in is calculated from total OTUs used in the Species-level analysis. Each percentage corresponds specifically to each row; taxa are presented in the highest taxonomic resolution achieved. In other words, taxa that fall within the category of another OTU have not been summed (e.g. OTU 1 g:Arctotheca was not summed with OTU 6 f:Asteraceae). All OTUs were used for Species- and Class-level analyses. [a]Denotes merged duplicate OTUs. [b]Separate analysis was also run with exclusion of this OTU. [c]Potential bait item (up to Family level).

complete dataset; Fig. 1) with the primary goal being to detect Little Penguin DNA and determine correspondence with known culprits, contextualising that data in the broader diet using the Chiar 16S and ITS2 datasets.

RESULTS

Detection of penguin in raven faeces

Using the penguin-specific primer, gel reads positively indicated penguin DNA (species *Eudyptula minor*) in six of the positive control samples (42.9%; $n = 14$, excluding the penguin tissue and maggot samples) and two samples in the optimised dataset (4.3%; $n = 47$). For NGS using mitochondrial 16S rRNA, one of the 14 positive control samples and three of 47 samples in the optimised dataset were excluded due to missing data. In four of the remaining 13 positive control samples analysed (30.8%), NGS detected penguin DNA. In the remaining 44 samples of the optimised dataset, NGS detected penguin DNA in eight samples (18.2%) from six individuals, split equally between ravens identified as Possible Culprits and Other Birds. Both NGS and penguin-specific primer methods matched in detecting penguin for three particular samples. The penguin-specific primer results indicated that NGS failed to detect penguin in three samples among the positive controls (and another sample from the optimised dataset which had missing data in the NGS) but NGS also detected penguin DNA in one positive control and seven optimised dataset samples not uncovered by the penguin-specific primer. Thus, penguin was detectable, although not always, regardless of the analysis used. Additionally, some 'Other Birds' had penguin detected in their diet. Both NGS and penguin-specific primer methods detected penguin DNA in both the positive control penguin tissue and maggot sample.

General raven diet

Mitochondrial 16S rRNA

Analysis of mitochondrial 16S rRNA reads from the NGS data for the optimised dataset revealed 32 OTUs across 44 of 47 samples from a range of taxa. Following exclusion of host and human DNA, the OTUs were resolved at the Class and Species levels (Table 2) to enable comparisons between individuals of different culprit status.

Table 2. List of detected taxa within the optimised dataset against mitochondrial 16S rRNA.

OTU	Kingdom	Phylum	Class	Order	Family	Genus	Species	Common name	% of samples detected
1	k:Eukaryota							k:Eukaryotes	9.09
2	k:Eukaryota	p:Arthropoda						p:Arthropods	11.36
3[a]	k:Eukaryota	p:Arthropoda	c:Insecta					c:Insects	4.55
4[a,b]	k:Eukaryota	p:Arthropoda	c:Insecta	o:Diptera	f:Tephritidae			f:Fruit Flies	4.55
5[a,b]	k:Eukaryota	p:Arthropoda	c:Insecta	o:Thysanoptera	f:Thripidae	g:*Thrips*	s:*unknown_Thrips*	s:UnknownThrips	43.18
6[a,b]	k:Eukaryota	p:Arthropoda	c:Malacostraca	o:Isopoda	f:Porcellionidae	g:*Porcellio*	s:*Porcellio_scaber*	s:Common Rough Woodlouse	2.27
7	k:Eukaryota	p:Chordata						p:Chordates	9.09
8[a,b]	k:Eukaryota	p:Chordata	c:Actinopteri		f:Sillaginidae	g:*Sillago*	s:*Sillago_flindersi*	s:Eastern School Whiting	4.55
9[a,b]	k:Eukaryota	p:Chordata	c:Actinopteri	o:Clupeiformes	f:Clupeidae	g:*Sardinops*	s:*Sardinops_sagax*	s:South American Pilchard	13.64
10[a,b]	k:Eukaryota	p:Chordata	c:Actinopteri	o:Clupeiformes	f:Engraulidae	g:*Engraulis*	s:*Engraulis_encrasicolus*	s:European Anchovy	9.09
11[a,b]	k:Eukaryota	p:Chordata	c:Actinopteri	o:Syngnathiformes	f:Mullidae	g:*Upeneichthys*	s:*Upeneichthys_vlamingii*	s:Southern Goatfish	2.27
12[a,b]	k:Eukaryota	p:Chordata	c:Actinopteri	o:Tetraodontiformes	f:Tetraodontidae	g:*Tetractenos*	s:*Tetractenos_glaber*	s:Smooth Toadfish	2.27
13[a,b]	k:Eukaryota	p:Chordata	c:Aves	o:Galliformes				o:Land Fowl	20.45
14[a,b,c]	k:Eukaryota	p:Chordata	c:Aves	o:Galliformes	f:Phasianidae	g:*Gallus*	s:*Gallus_gallus*	s:Chicken	31.82
15[d]	k:Eukaryota	p:Chordata	c:Aves	o:Passeriformes	f:Corvidae	g:*Corvus*		g:Corvids	100.00
16[d]	k:Eukaryota	p:Chordata	c:Aves	o:Passeriformes	f:Corvidae	g:*Corvus*	s:*Corvus_macrorhynchos*	s:Large-billed Crow	100.00
17[a,b]	k:Eukaryota	p:Chordata	c:Aves	o:Passeriformes	f:Estrildidae	g:*Taeniopygia*	s:*Taeniopygia_guttata*	s:Zebra Finch	2.27
18[a,b,c]	k:Eukaryota	p:Chordata	c:Aves	o:Procellariiformes	f:Procellariidae	g:*Ardenna*		g:Seabirds	4.55
19[a,b]	k:Eukaryota	p:Chordata	c:Aves	o:Sphenisciformes	f:Spheniscidae	g:*Eudyptula*	s:*Eudyptula_minor*	s:Little Penguin	18.18
20[a,b]	k:Eukaryota	p:Chordata	c:Lepidosauria	o:Squamata	f:Scincidae			f:Skinks	2.27
21[a,b]	k:Eukaryota	p:Chordata	c:Lepidosauria	o:Squamata	f:Scincidae	g:*Carinascincus*	s:*Carinascincus_metallicus*	s:Metallic Cool-Skink	27.27
22[a,b]	k:Eukaryota	p:Chordata	c:Lepidosauria	o:Squamata	f:Scincidae	g:*Cyclodomorphus*		g:Skinks	2.27
23[a,b]	k:Eukaryota	p:Chordata	c:Lepidosauria	o:Squamata	f:Scincidae	g:*Saproscincus*	s:*Saproscincus_mustelinus*	s:Weasel Skink	11.36
24[a]	k:Eukaryota	p:Chordata	c:Mammalia					c:Mammals	6.82
25[a,b,c]	k:Eukaryota	p:Chordata	c:Mammalia	o:Artiodactyla	f:Bovidae			f:Bovid	4.55
26[a,b,c]	k:Eukaryota	p:Chordata	c:Mammalia	o:Artiodactyla	f:Bovidae	g:*Bos*	s:*Bos_taurus*	s:Cattle	15.91
27[a,b,c]	k:Eukaryota	p:Chordata	c:Mammalia	o:Artiodactyla	f:Bovidae	g:*Ovis*	s:*Ovis_aries*	s:Sheep	22.73

(continued)

Table 2. (continued)

OTU	Kingdom	Phylum	Class	Order	Family	Genus	Species	Common name	% of samples detected
28[a,b,c]	k:Eukaryota	p:Chordata	c:Mammalia	o:Artiodactyla	f:Suidae	g:*Sus*	s:*Sus_scrofa*	s:Pig	25.00
29[a,b]	k:Eukaryota	p:Chordata	c:Mammalia	o:Diprotodontia	f:Macropodidae	g:*Wallabia*	s:*Wallabia_bicolor*	s:Swamp Wallaby	4.55
30[a,b]	k:Eukaryota	p:Chordata	c:Mammalia	o:Diprotodontia	f:Phalangeridae	g:*Trichosurus*	s:*Trichosurus_vulpecula*	s:Common Brushtail	18.18
31[a,b]	k:Eukaryota	p:Chordata	c:Mammalia	o:Lagomorpha	f:Leporidae	g:*Oryctolagus*	s:*Oryctolagus_cuniculus*	s:Rabbit	13.64
32[d]	k:Eukaryota	p:Chordata	c:Mammalia	o:Primates	f:Hominidae	g:*Pan*	s:*Pan_troglodytes*	s:Chimpanzee	4.55

The percentage of samples that each taxon was detected in is calculated from total OTUs used in the Species-level analysis. Each percentage corresponds specifically to each row; taxa are presented in the highest taxonomic resolution achieved. In other words, taxa that fall within the category of another OTU have not been summed (e.g. OTU 21 s:*Carinascincus metallicus* was not summed with OTU 20 f:Scincidae). [a]OTU used in the Class-level analysis. [b]OTU used in the Species-level analysis. [c]Potential bait item (up to Family level). [d]OTU excluded from all analyses.

Across samples, mammals (class Mammalia) were detected most frequently (59.09% of 44 samples) followed by birds (class Aves; 45.45%) and insects (class Insecta; 45.45%), reptiles (class Lepidosauria; 38.64%), ray-finned fish (class Actinopteri; 22.73%) and crustaceans (class Malacostraca; 2.27%). In addition to Little Penguin, NGS appeared to detect Short-tailed Shearwaters (genus *Ardenna*) – the only commonly occurring species of the genus on Phillip Island, where it is highly abundant. Other native species of interest detected by NGS (acknowledging that species determined by OTUs may not be exact and only indicative) included Swamp Wallaby *Wallabia bicolor*, Common Brushtail Possum *Trichosurus vulpecula*, Metallic Cool-skink *Carinascincus metallicus*, Weasel Skink *Saproscincus mustelinus*, Southern Goatfish *Upeneichthys vlamingii* and Smooth Toadfish *Tetractenos glaber*.

Using presence/absence data at the Class level, PERMANOVA revealed no significant differences for OTUs across Culprit Status (*Pseudo-*$F_{2,9}$ = 0.897, P = 0.517), between Sex (*Pseudo-*$F_{1,9}$ = 0.433, P = 0.618), Culprit Status*Sex (*Pseudo-*$F_{1,9}$ = 1.681, P = 0.228) and Date (*Pseudo-*$F_{1,9}$ = 1.122, P = 0.344). The random factor (ID) was significant, suggesting substantial between-individual variation (*Pseudo-*$F_{29,9}$ = 4.014, P = 0.001). A second analysis at the Species level revealed no significant differences with Culprit Status (*Pseudo-*$F_{2,9}$ = 1.062, P = 0.394), Sex (*Pseudo-*$F_{1,9}$ = 0.701, P = 0.558), Culprit Status*Sex (*Pseudo-*$F_{1,9}$ = 1.350, P = 0.249) or Date (*Pseudo-*$F_{1,9}$ = 0.709, P = 0.526). The random factor (ID) was again significant (*Pseudo-*$F_{29,9}$ = 2.746, P = 0.001).

Chiar 16S

For NGS using Chiar 16S on the complete dataset, data were successfully obtained for 94 of 109 samples (15 were excluded due to missing data). Based on Marquina *et al.* (2019), this primer was expected to be specific for insect items in the diet. However, NGS detected several non-insect OTUs including those of classes Aves and Mammalia (Table 3). Unexpectedly, Chiar 16S was also able to detect Little Penguin specifically in four samples at the Species level, three of which had also been detected by NGS of mitochondrial 16S rRNA, and one of which had been detected by the penguin-specific primer but was excluded due to missing data in the mitochondrial 16S rRNA NGS.

Table 3. List of detected taxa within the complete dataset against Chiar16S.

OTU	Kingdom	Phylum	Class	Order	Family	Genus	Species	Common Name	% of samples detected
1[a]	k:Eukaryota							k:Eukaryotes	12.77
2[b,c]	k:Eukaryota	p:Annelida	c:Clitellata	o:Haplotaxida	f:Lumbricidae	g:*Aporrectodea*	s:*Aporrectodea_trapezoides*	s:Earthworm	1.06
3[b,c]	k:Eukaryota	p:Annelida	c:Clitellata	o:Haplotaxida	f:Megascolecidae			f:Earthworms	1.06
4[b]	k:Eukaryota	p:Arthropoda	c:Collembola					c:Springtails	17.02
5[b,c]	k:Eukaryota	p:Arthropoda	c:Collembola	o:Poduromorpha	f:Hypogastruridae	g:*Ceratophysella*		g:Springtails	1.06
6[a]	k:Eukaryota	p:Arthropoda						p:Arthropods	13.83
7[a,b]	k:Eukaryota	p:Arthropoda	c:Insecta					c:Insects	7.45
8[a,b,c]	k:Eukaryota	p:Arthropoda	c:Insecta	o:Diptera				o:Two-winged Fly	4.26
9[b,c]	k:Eukaryota	p:Arthropoda	c:Malacostraca	o:Isopoda	f:Porcellionidae	g:*Porcellio*	s:*Porcellio_scaber*	s:Common Rough Woodlouse	6.38
10[b,c]	k:Eukaryota	p:Chordata	c:Actinopteri	o:Clupeiformes	f:Clupeidae	g:*Sardinops*	s:*Sardinops_sagax*	s:South American Pilchard	5.32
11[b,c]	k:Eukaryota	p:Chordata	c:Actinopteri	o:Syngnathiformes	f:Mullidae	g:Upeneichthys	s:*Upeneichthys_vlamingii*	s:Southern Goatfish	1.06
12[d]	k:Eukaryota	p:Chordata	c:Aves	o:Passeriformes	f:Corvidae	g:*Corvus*		g:Corvids	100.00
13[b,c]	k:Eukaryota	p:Chordata	c:Aves	o:Sphenisciformes	f:Spheniscidae	g:*Eudyptula*	s:*Eudyptula_minor*	s:Little Penguin	4.26
14[b,c]	k:Eukaryota	p:Chordata	c:Lepidosauria	o:Squamata	f:Scincidae	g:*Carinascincus*		g:Cool Skinks	9.57
15[b,c]	k:Eukaryota	p:Chordata	c:Lepidosauria	o:Squamata	f:Scincidae	g:*Pseudemoia*		g:South-eastern Australian Skinks	1.06
16[b,c]	k:Eukaryota	p:Chordata	c:Lepidosauria	o:Squamata	f:Scincidae	g:*Saproscincus*	s:*Saproscincus_mustelinus*	s:Weasel Skink	4.26
17[b,c,e]	k:Eukaryota	p:Chordata	c:Mammalia	o:Artiodactyla	f:Bovidae	g:*Bos*	s:*Bos_taurus*	s:Domestic Cow	3.19
18[b,c]	k:Eukaryota	p:Chordata	c:Mammalia	o:Diprotodontia	f:Macropodidae	g:*Wallabia*	s:*Wallabia_bicolor*	s:Swamp Wallaby	2.13
19[b,c]	k:Eukaryota	p:Chordata	c:Mammalia	o:Diprotodontia	f:Phalangeridae	g:*Trichosurus*	s:*Trichosurus_vulpecula*	s:Common Brushtail	4.26
20[b,c,e]	k:Eukaryota	p:Chordata	c:Mammalia	o:Lagomorpha	f:Leporidae	g:*Oryctolagus*	s:*Oryctolagus_cuniculus*	s:Rabbit	9.57
21[d]	k:Eukaryota	p:Chordata	c:Mammalia	o:Primates	f:Hominidae	g:*Homo*	s:*Homo_sapiens*	s:Human	4.26

The percentage of samples that each taxon was detected in is calculated from total OTUs used in the Species-level analysis. Each percentage corresponds specifically to each row; taxa are presented in the highest taxonomic resolution achieved. In other words, taxa that fall within the category of another OTU have not been summed (e.g. OTU 5 g:Ceratophysella was not summed with OTU 4 c:Collembola). [a]Merged duplicate OTU. [b]OTU used in the Class-level analysis. [c]OTU used in the Species-level analysis. [d]OTU excluded from all analyses. [e]Potential bait item (up to Family level).

Across samples, springtails (class Collembola) were detected most frequently (18.09% of 94 samples) followed by mammals (class Mammalia; 17.02%), reptiles (class Lepidosauria; 12.77%), insects (class Insecta; 10.64%), crustaceans and ray-finned fish (class Malacostraca and Actinopteri, respectively; both 6.38%), birds (class Aves; 4.26%) and annelid worms (class Clitellata; 2.13%).

NGS analysing Chiar 16S for the complete dataset revealed 21 OTUs across 94 of 109 samples from a range of taxa, before exclusion of human and host DNA (Table 3). Using presence/absence data at the Class level, PERMANOVA revealed no significant differences across Culprit Status (*Pseudo-*$F_{2,36}$ = 0.878, P = 0.579), between Sex (*Pseudo-*$F_{1,36}$ = 0.626, P = 0.671), Culprit Status*Sex (*Pseudo-*$F_{2,36}$ = 0.868, P = 0.508) and Date (*Pseudo-*$F_{1,36}$ = 1.302, P = 0.298). The random factor (ID) was not significant (*Pseudo-*$F_{51,36}$ = 1.313, P = 0.070). A second analysis at the Species level revealed no significant differences for Culprit Status (*Pseudo-*$F_{2,36}$ = 0.948, P = 0.550), Sex (*Pseudo-*$F_{1,36}$ = 0.956, P = 0.452), Culprit Status*Sex (*Pseudo-*$F_{2,36}$ = 0.576, P = 0.691) or Date (*Pseudo-*$F_{1,36}$ = 0.789, P = 0.556). The random factor (ID) was not significant (*Pseudo-*$F_{51,36}$ = 1.260, P = 0.126).

ITS2

For NGS using ITS2 on the complete dataset, data were successfully obtained for 99 of 109 samples (10 were excluded due to missing data). Based on Cheng *et al.* (2016) this primer was expected to be specific only for plant items in the diet. NGS analysing ITS2 for the complete dataset revealed 50 OTUs across 99 of 109 samples from a range of taxa (Table 1). These were resolved into Sub-class and Species levels to enable comparisons (Table 1).

Sub-classes Caryophyllanae and Lilianae were detected most frequently in raven samples (96.97% and 79.80% of 99 samples, respectively), followed by sub-classes Rosanae (35.35%), Asteranae (32.32%), Pinidae (3.03%) and Proteanae (2.02%). Bower Spinach *Tetragonia implexicoma*, which is a dominant vegetation within the Little Penguin colony habitat, contributed immensely towards the Caryophyllanae figure, being detected in almost all samples (91.92%; Table 1). Bower Spinach was detected in just over twice as many samples as the next most frequently occurring OTUs, which were from the genera *Triticum* (45.45%), *Bromus* (44.44%), *Lolium* (44.44%) and *Chenopodium* (42.42%).

Using presence/absence data at the Class (i.e. sub-class) level, PERMANOVA revealed no significant differences across Culprit Status (*Pseudo-*$F_{2,39}$ = 0.883, P = 0.584), between Sex (*Pseudo-*$F_{1,39}$ = 0.322, P = 0.784), Culprit Status*Sex (*Pseudo-*$F_{2,39}$ = 0.685, P = 0.556) and Date (*Pseudo-*$F_{1,39}$ = 0.364, P = 0.775). The random factor (ID) was not significant (*Pseudo-*$F_{53,39}$ = 1.145, P = 0.235). A second analysis at the Species level revealed no significant differences for Culprit Status (*Pseudo-*$F_{2,39}$ = 1.026, P = 0.431), Sex (*Pseudo-*$F_{1,39}$ = 0.783, P = 0.663), Culprit Status*Sex (*Pseudo-*$F_{2,39}$ = 0.856, P = 0.561) or Date (*Pseudo-*$F_{1,39}$ = 0.960, P = 0.459). The random factor (ID) was significant (*Pseudo-*$F_{53,39}$ = 1.275, P = 0.002).

DISCUSSION

Can DNA metabarcoding identify penguin in the diet of ravens?

Penguin DNA signatures were detected in extracted raven faecal samples using both NGS (with mitochondrial 16S rRNA and Chiar 16S primers) and gel reads using a penguin-specific primer. Although we lacked an effective blocking primer (BLOCK) to assist in masking high host gDNA for mitochondrial 16S rRNA and Chiar 16S analyses, insight was still gained on raven diet from the OTU information provided. We found that Chiar 16S also PCR-amplified successfully against raven faecal samples but was not specific for insects as was expected. While not directly comparable to mitochondrial 16S rRNA due to the use of different datasets, Chiar 16S detected two classes (Clitellata and Collembola) in the same samples which featured in both analyses yet were not detected by mitochondrial 16S rRNA. ITS2 also PCR-amplified successfully against raven faecal samples and as expected was specific for plant taxa, assisting in documenting that vegetation forms part of the general raven diet.

Known culprit individual ravens (DC) were 'missed' insofar as penguin was not detected in their diets by either 16S rRNA or Chiar 16S, probably due to the short latency of DNA and episodic consumption of penguin (Culliney *et al.* 2012, Simonová *et al.* 2016, Thuo *et al.* 2019). However, penguin was detected in the faecal samples of other individuals not previously identified as culprits preying on penguins. While it is possible these birds may be culprits not

identified by observation methods, it is also possible that they are not true culprits (false positives). Detection may also have been due to the scavenging of penguin carcasses or secondary consumption of, for example, maggots feeding on dead penguins rather than preying upon penguins. Some Australian corvids are notorious scavengers of necromass from coastal environments (Schlacher *et al.* 2013) and penguin carcasses are not uncommon around colonies (Dann 1992).

We note that for some positive control samples, penguin was not detected. This is probably due to insubstantial amounts or lack of penguin being consumed by the captive bird, leading to insufficient DNA being available for detection in some of those samples. Although not implemented in this study to avoid stressing the captive bird, sample collection at more frequent intervals (in conjunction with constant video monitoring) would have allowed us to better determine the potential cause of the lack of penguin detection in faecal samples (e.g. insufficient digestion time and/or insufficient consumption of penguin). However, the positive control samples fulfilled the purpose of being from a bird observed consuming penguin, and having penguin detected in a number of those samples confirmed the validity of penguin detections in the complete and optimised datasets.

Overall, the diet of culprit ravens included items other than penguin, indicating that individual ravens were not specialist penguin consumers. Penguin eggs and chicks are seasonal in terms of availability, and ravens are known to target penguin eggs and young during the penguin breeding season (Ekanayake *et al.* 2015b). Ravens which had consumed penguin in most cases had also consumed other items that could not be linked to direct or secondary consumption of penguin (SOM Tables S2–S4, Appendix S2), suggesting a somewhat generalised raven diet even at times when penguin had been consumed.

The diet of ravens

Prey items were successfully identified using raven faeces and metabarcoding. Generally, the range of items detected falls in line with stomach contents analyses conducted by Rowley and Vestjens (1973) on inland-residing Little Ravens. Marine-based items (not reported by Rowley & Vestjens 1973) were also detected which were consistent with raven foraging behaviour observed on Phillip Island beaches (Swinburne & Jessop 2005). Classification into marine and terrestrial prey was not attempted here because coasts experience ecological subsidies in the form of terrestrial inputs (e.g. beach-cast terrestrial animals) and marine inputs (e.g. beach-cast fish; Beasley *et al.* 2012, Schlacher *et al.* 2013). Thus, terrestrial food may have been sourced from the shoreline. It is clear from this study that raven diet is broad, opportunistic, and spans terrestrial and shoreline food sources.

Whereas some faecal metabarcoding studies of predator diet have revealed predation as a key threat to sensitive prey (Oja *et al.* 2017), aside from the target of penguin, we did not detect any species of conservation concern in raven diet via NGS. Based on information available on Little Raven diet (Rowley & Vestjens 1973, Swinburne & Jessop 2005, Lill & Hales 2015), we expected invertebrates to feature prominently; insects were of equal second greatest frequency of occurrence across samples for NGS of mitochondrial 16S rRNA (after mammals and equal to birds). That insects were not the most frequently occurring group across samples may be due to a difference in detection between morphological and DNA analysis methods (e.g. Mumma *et al.* 2016, Zarzoso-Lacoste *et al.* 2016, de Sousa *et al.* 2019). It is also possible that the higher and equal occurrences of mammal and bird items detected (respectively) may be due to faecal samples containing traces of bait from capture, and we acknowledge that certain dietary items (e.g. chicken, sheep, cow, pig) could possibly contribute to these results and hence we interpret these detections with caution. However, other plausible explanations exist for their frequent detection. There are several farming properties on Phillip Island where these dietary items could be found, and it is possible those items were scavenged on farmland by ravens (Rowley & Vestjens 1973). Certainly, Little Ravens are seen on farms at Phillip Island (L.X.L. Tan pers. obs.). In addition, the Summerland Peninsula and Penguin Parade draw many tourists to the peninsula throughout the year (PINP Annual Report 2018–2019), making human food waste available to ravens; even away from the penguin colony there are numerous urbanised, well-populated areas on the island and its surrounds where ravens could obtain human food.

Similarly, the detection of items from the wheat family (genus *Triticum*) in almost half our samples may have come from human sources, including

the direct feeding of bread to ravens by tourists or residents (Swinburne & Jessop 2005, PINP Annual Report 2018–2019), but it may also be explained by the inclusion of bread used as bait in our traps. Hence, we interpret this result with caution. Commonly occurring vegetation within the Summerland Peninsula was detected in Little Raven diet, including Bower Spinach, items within the genus *Chenopodium* (probably including Seaberry Saltbush *Chenopodium candolleanum*) and various wheats and grasses. We expected Seaberry Saltbush to feature prominently in the plant diet, as several samples collected were stained red/purple from berries presumably from this plant (L.X.L. Tan pers. obs.), supplementing previous observations by Swinburne and Jessop (2005). Bower Spinach also produces berries after flowering, so it is possible the coloration was observed from spinach and not saltbush berries. Regardless, we did not expect it to feature as prominently within plant sequencing reads as it did. Other explanations for the frequency and abundance in which it was detected may include that Bower Spinach is simply ingested as a by-product when foraging for invertebrates, secondary consumption, or that its detectability using the ITS2 and NGS methods is very sensitive to the presence of even trace amounts of material (although the latter is less likely).

Limitations of faecal DNA metabarcoding in the study system

Corvids hunt and scavenge other animals (Rowley & Vestjens 1973, Schlacher *et al.* 2013, Cardilini *et al.* 2012, Fielding *et al.* 2020, Sazima 2020). Certain items detected were almost certainly scavenged (e.g. shearwaters; Swinburne & Jessop 2005, rabbit and swamp wallaby), but smaller items (e.g. skinks and finches) may have been preyed upon live (see Cardilini *et al.* 2012). Some fish species detected may be the result of secondary consumption from scavenging on penguin or shearwater remains, or preying on chicks that had been fed fish by an adult (e.g. pilchards *Sardinops sagax*; Montague & Cullen 1988, Dann *et al.* 2000). Based on their size and ecology, other items (such as the large proportion of springtails detected) are also likely to be the result of secondary consumption or may have been collected on the faecal samples themselves (i.e. colonised the samples after deposition and before collection). Ravens may consume dipterous larvae associated with carrion (Rowley & Vestjens 1973). Given that we were able to successfully detect penguin in the control maggot samples, associating detection of penguin with direct consumption of penguin (and similarly attributing culprit status based solely on positive detections) should be done with caution.

Although the snapshots which faecal metabarcoding provides can be exceptionally useful in determining specific diet over time (Gable *et al.* 2018), episodic and possibly infrequent consumption of highly calorific or nutritious prey, such as penguin eggs or young, may mean consumption events are missed using this method. This is supported here by some cases where multiple samples were obtained for the same individual, and for which dietary items often varied among samples (SOM Figs S2, S4 and S6, Appendix S2). This may be especially true if satiated individuals are less motivated to enter baited traps (Weatherhead & Greenwood 1981). However, poor detection could arguably be overcome through more regular and frequent sampling during peak penguin breeding periods. Trapping of ravens and the ability to obtain faecal samples was primarily conducted in July and August, before typical peak penguin-breeding (Reilly & Cullen 1981, Ekanayake *et al.* 2015b) and when scavenging on penguin remains by ravens may have been more likely. Raven trapping was limited during the penguin breeding season as we focused on penguin burrow monitoring to identify depredation events (Tan *et al.* 2021, 2022). Considering that corvid faecal samples tend to reflect diet from a relatively short time frame and food items may be passed within 18–26 h after consumption (e.g. Culliney *et al.* 2012, Simonová *et al.* 2016), more penguin might have been identified in the diet if trapping had been conducted more intensively during the penguin breeding season (particularly among known culprit individuals). However, ravens preying upon early-age penguin eggs, which might contain little DNA, might remain undetected, although it is worth noting that some ravens appeared to return to burrows and take eggs near the time of hatching (K.B. Ekanayake unpubl. data). Metabarcoding detection rates may also vary depending on the prey species consumed (Thuo *et al.* 2019), and taking possible false negatives into account (i.e. for molecular methods, a prey item that has been consumed but not detected; Mumma *et al.* 2016), we cannot claim diet is

unambiguously resolved. In terms of detecting penguin specifically, understanding the latency period and quantities of penguin items required for successful detection (*sensu* Thuo *et al.* 2019) would assist in determining the frequency of any false negatives.

Despite these shortfalls, we demonstrate success in detecting penguin DNA in birds not previously identified as having preyed on penguins (i.e. culprits). More frequent sampling would assist in mitigating the problem of false-negative detections (Mumma *et al.* 2016). Additionally, new methods (e.g. use of bacterial biomarkers) may help distinguish whether signatures are from scavenged tissue or live prey (Muletz-Wolz *et al.* 2021). If a successful host-blocking primer can be established for Little Raven, the improved effectiveness of NGS output (i.e. greater number of on target reads) and the use of bacterial biomarkers would enable differentiation between scavenged and live prey consumption (Vestheim & Jarman 2008, Muletz-Wolz *et al.* 2021), and may provide a compelling method for establishing diet over time in birds (e.g. Gable *et al.* 2018, Voelker *et al.* 2020). In doing so, such analyses could establish whether it is abundance of Little Penguin or other spatial factors that draw ravens to the Summerland Peninsula during peak penguin breeding periods (Bastille-Rousseau *et al.* 2011, Ekanayake *et al.* 2015b, Ando *et al.* 2016).

CONCLUSIONS

Faecal metabarcoding provided a way of non-invasively characterising aspects of predator diets. NGS using mitochondrial 16S rRNA and Chiar 16S primers detected a number of native vertebrate species ranging from large mammals (e.g. Swamp Wallabies and Brush-tailed Possums) to smaller species such as skinks, which in addition to contextualising penguin in raven diet, could potentially be useful in establishing occurrence of prey species at a location.

However, faecal metabarcoding does not currently present a way to unambiguously distinguish between burrow-raiding culprit ravens and ravens which do not raid burrows, assuming the distinction exists. First, it does not distinguish between ravens which have scavenged upon penguin carcasses (i.e. 'false positives' in terms of identifying culprit ravens), and thus culprit status cannot confidently be assigned to ravens on the basis of DNA detection of penguin in diet. Secondly, it only provides episodic information that may miss penguin consumption events due to the short retention time of penguin DNA in the raven gut. Finally, ravens that prey upon early-age penguin eggs containing little DNA may also be missed, resulting in 'false negatives' in terms of identifying culprits (Mumma *et al.* 2016). In conclusion, in this system we suggest the method only be used in a supplemental fashion to direct observation with respect to identifying which ravens are preying upon Little Penguins.

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AUTHOR CONTRIBUTIONS

Laura X. L. Tan: Conceptualisation; investigation; methodology; data curation; formal analysis; funding acquisition; project administration; writing – original draft; writing – review and editing. **Han M. Gan:** Conceptualisation; methodology; software; data curation; investigation; supervision; visualisation; resources; writing – review and editing. **Wouter F.D. van Dongen:** Conceptualisation; methodology; supervision; writing – review and editing. **Peter Dann:** Conceptualisation; supervision; funding acquisition; resources; writing – review and editing. **Duncan R. Sutherland:** Conceptualisation; funding acquisition; writing – review and editing; supervision; resources. **Michael A. Weston:** Conceptualisation; methodology; investigation; formal analysis; supervision; funding acquisition; resources; writing – original draft; writing – review and editing.

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflict of interest.

Data Availability Statement

Raw data are available via Dryad at: https://doi.org/10.5061/dryad.jh9w0vtj4

ETHICAL NOTE

Research was conducted in accordance with Deakin University Animal Ethics approvals A08-2011, B19-2012 and B07-2015; Department of Environment, Land, Water and Planning permits 10 005 849, 10 006 360 and 10 007 531; and Australian Bird and Bat Banding Scheme Authority 1763.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Appendix S1. Custom primer details and obtaining positive control samples.

Appendix S2. Additional analyses and results.