December 10th, 2021

Dear Prof. Mark Burgman and anonymous reviewer,

We sincerely thank our reviewer, and Prof. Mark Burgman, editor, for your time in considering our manuscript for publication with Conservation Biology. We are pleased to resubmit a thoroughly revised manuscript and a detailed response to the reviewer’s concerns. We hope that this effort provides a high level of confidence and clarity in our methods and work.

We acknowledge that exploring intraspecific variation is uncommon on metabarcoding data, but it is valid, and it can be informative when conservative. We provide several in text citations throughout for similar applications. Ultimately, the reviewer needed more clarity and detail to ensure that we had been conservative, and cognisant of the limitations of DNA metabarcoding. We thus endeavoured to add all information requested by the reviewer. We also emphasize both in this letter and in the manuscript that this type of analysis should be used to motivate a deeper dive into the research nexus of population genetics and environmental sampling, and state that our results highlight a clear need for further assays and ongoing monitoring.

The reviewer’s first concern regarded the amount of overlap in taxa identified simultaneously by DNA metabarcoding vs. traditional morphometric hard parts. We clarified text on the completion of duplicate PCRs and that scat samples were indeed thoroughly mixed prior to sub-sampling. We also performed a more rigorous single-step fusion PCR for sequencing and not two-step PCR which is more susceptible to cross-contamination. If any duplicates were positive for penguins, a sub-sample from the original DNA extract was submitted for sequencing. More than 1/3-1/2 overlap of samples between DNA and morphological assays is uncommon in our system and biologically improbable. We add this context and key references to support this argument in the introduction and the discussion. Seals, for instance, do not digest these tissues at the same rate, making these two methods near-separate assays of the same seal.

The reviewer’s second concern arose due to our brevity in explaining how we jumped from our OTU analysis to perform the polymorphism analysis, which we now clarify in our methods. Most of these issues came up again in the reviewer’s third concern. In addition, we increased the stringency of our criteria for including a sequence in haplotype polymorphism analysis, to rule out intrusion of error beyond reasonable doubt. Our previous threshold for haplotype polymorphism analysis had been > 1% of the total sequence abundance in each sample (the commonly accepted error rate for Illumina Miseq) and included 12 sequences. We increased that threshold to > 7.5% and include only 9 of the most abundant sequences, from only the 6 faecal samples that contained abundant DNA. For clarity, and this is noted in the results and discussion, the remaining four samples contained small quality-controlled quantities of DNA, representing likely an older meal or traces of a meal of penguin(s) and we searched these samples for haplotypes identified from the more abundant samples, but we chose conservatively not to add any unique haplotypes from the less abundant samples to the analysis. This step therefore excluded over a thousand sequences and that followed our typical procedures for quality control and sequence filtering that had already excluded 20,000+ sequences and clarified in Methods.

One last concern of the reviewer’s was the existence of any comparable haplotypes, and we have identified one of the main haplotypes extracted from muscle tissue from a penguin carcass found at the time of the study on Phillip Island, and the same haplotype was found in a whole genome on GenBank, and from two other whole genomes from GenBank described in our supplement. Tsuji and co-authors do mention that the presence of reference haplotype material is an existing problem for many ecosystems and study species.

We thus apply a developed polymorphism analysis approach to a novel question and system, using very stringent selection of sequences, and provide tangible recommendations for next steps in monitoring and managing this wildlife conflict scenario. We believe there can be no harm in reporting on the genetic diversity observed in our samples after having undergone very stringent filtering and thresholds for inclusion in analyses, especially for a conserved gene and in such a desperate context, where researchers and managers are clutching at straws of information – such as how many scats containing feathers does one seal pass after eating one penguin (Goldsworthy’s and co-authors’ 2019 report cited in the manuscript did research this arduous question, the answer was 5, but the applicability of that number in the field is unclear to us who work in this field). Managers, including several co-authors, need other methods and other data, such as rigorous genetic information on this conflict as well. This paper will inspire growth and development of wildlife forensic and monitoring techniques not only in this context.

We sincerely hope that the reviewer, editor, and readership will appreciate the balance we have aimed to achieve in the level of care taken to control for any sources of error while presenting novel and impactful research. One offshore island little penguin population in southeastern Australia, for which we obtained evidence of fur seal predation, has crashed this year during the time this paper has been in review, and this paper will be instrumental in determining monitoring and management solutions. This paper is positioned to be of significant relevance to conservation practitioners faced with rapid species declines, species redistributions under climate change, and growing species conflicts in response to human-altered ecosystems and food webs.

In our resubmission, we include both a cleaned MS version and supplementary information document, as well as both with tracked changes visible. We understand this is a long response as we take the reviewer’s concerns with utmost seriousness and attention to detail. We provide a detailed response to comments in bullets below this letter.

Thank you very much for your time,

Dr. Natasha Hardy

RESPONSE TO SPECIFIC CONCERNS:

Concern #1 re overlap in DNA and hard-parts detected in samples:

* To avoid confusion, we changed language to use “apply” and we clarify the scope being “overall” as opposed to sample-by-sample (Abstract line 11, Introduction line 90).
* We add the following text to guide readers expectations of our study and assaying techniques (Introduction lines 91–96): “Due to known biological and methodological differences in dietary information (Casper et al., 2007; Tollit et al., 2009), and differences in the quantities of DNA obtained from hard-parts compared to soft tissues (McDonald & Griffith, 2011), we consider these techniques complementary. Specifically, (i) we compare overall seabird and little penguin detection rates across groups of samples, rather than sample-by-sample; …”
* To ensure the homogenisation and field sampling steps are clear, we replaced the former Methods text from lines 135–138 “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 Eppendorf tubes.” With text in lines 116–120 “Whole scats were thoroughly mixed with individual disposable spatulas at point of collection and a 2 mL subsample was taken from each field-homogenized scat for DNA-based analyses of prey remains. The remaining whole scats were collected for analyses of morphological prey remains, using individual, zip-lock bags.”
* Samples were indeed screened in duplicate using diagnostic endpoint PCRs which we noted formerly in lines 162–63, however we were previously too brief in describing what happened afterwards. We have clarified text in Methods line 135–138: “In total, 99 faecal DNA sample extracts at two DNA concentrations (neat and 1:10 dilutions), as well as extraction blanks (n = 5), PCR blanks (n = 4), and positive controls (n = 2) were screened in duplicates by diagnostic endpoint PCR (dPCR) using the Bird12sa/h assay (Cooper, 1994) (Table S1 & S2, Appendix S1).” And we clarified the following information also, lines 138–142: “Duplicate dPCR products were run on 1.5% agarose gels to determine the presence/absence of amplified target bird DNA in each duplicate. A total of 32 samples showed target amplicons in both or one duplicate, but not in the extraction and PCR controls. We sequenced each of the 32 samples that tested positive for birds, and two extraction blanks and one positive control (n = 35 samples for sequencing).”
* We clarified and edited our sequence quality-control and filtering procedures in Methods lines 149–161: “We used Geneious R8.1.5 (Kearse et al., 2012) to merge the paired-end sequences (2x ~150 bp fragments, with overlap of 70 bp) and retain only those with exact flanking sequences – MID tags, primers and adapters. Primers, adapters, and tags were removed to leave the complete target sequences in each sample. These were quality filtered and clustered into molecular operational taxonomic units (OTUs) using *UPARSE* algorithm and performed in *USEARCH* (Edgar, 2010; Edgar & Flyvbjerg, 2015). Low abundance sequences (below a threshold of 1% of the total abundance of all unique sequences) were removed to reduce the occurrence of sequencing error and chimeras, and sequences were then clustered using a 97% similarity criterion (similar to Berry et al., 2017). Thus 7370 unique seabird DNA sequences, representing a total of 64,700 disaggregated bird sequences, were parsed to standard sequence filtering and OTU clustering pipeline (with cluster size threshold value of 73), resulting in 47,478 filtered sequences across 99 samples, and these were clustered to 5 OTUs.”
* We respectfully disagree with the reviewer’s comment that hard-parts are a good source of DNA, this point risks oversimplifying of a complex body of knowledge and certainly we have not had the good fortune to easily extract DNA from such tissues (neither did McDonald & Griffith 2011 and others such as Granquist and co-authors have highlighted this issue). In our extensive experience working with large mammalian and fish predators, as well as ancient DNA – bones and feathers are not great sources of DNA, even less so in the presence of ‘fresher DNA’ that are preferentially extracted and amplified. Specialised DNA extraction techniques have been developed to obtain DNA from these keratin, sclerotin or chitin-based tissues (McDonald & Griffith 2011). Additionally, we target relatively short and common mitochondrial DNA sequences, these tend to be more present in soft tissues such as blood and organs (Discussion, lines 297–312).
* To clarify how our work is seated within that context, we have added a new paragraph (Discussion, lines 297–312): “While both metabarcoding and morphological methods provided statistically similar results, we emphasize that these represent complementary but quasi-independent assays of predation. Different detection rates reported between these methods in this study are also common across a broad range of predator and prey taxa (Tollit et al., 2009; Zarzoso-Lacoste et al., 2013). Many methodological and biological factors differentially affect the recovery of diagnostic genetic and morphological materials. In another pinniped species, prey soft tissues had a gut passage rate of 48 hours and up to 7 days for hard-parts (Tollit et al., 2009). Soft tissues may contain greater DNA quantities and concentrations, particularly mitochondrial (Mumma et al., 2016; Granquist et al., 2018), and could be preferentially amplified compared to DNA available from hard-parts, (i.e., feather, fur, or bone). DNA from these tissues is of poor quality and requires a different process for extraction (McDonald & Griffith, 2011; Rothe & Nagy, 2016). Typically, studies report higher detection rates from genetic assays compared to morphological analyses (Egeter et al., 2015; Mumma et al., 2016), but the lower detection rates here are consistent with other pinniped studies (Tollit et al., 2009) and with over-representation of feathers across fur seal scats (Goldsworthy et al., 2019), explaining the higher detection rate from these hard-parts.”
* We trust that this additional discussion and context also assuages the reviewers concern with an overlap of less than half the samples where both methods detected the same taxon. We note that this level of overlap and even less is very common in the literature, especially in our study system. For example, and we quote Tollit et al. (2009) finding “hard parts identifying the same prey in 65% of 213 DNA prey occurrences. In both regions, rankings between the two identification methods were similar, but the relative proportions of prey species occurrences in the diet were very different. We believe our results provide further evidence that hard parts found in scats are from a composite of many past meals (shown to be up to 7 d when eating Gadidae and even longer if cephalopods are consumed; Tollit et al. 2003), whereas prey present in scat soft-part matrix represent only the most recent feeding events (estimated to be diet over one to two days by both Deagle et al. [2005b] and Casper et al. [2007a]).” Additionally, Tollit et al. (2009) also found that only 20% of scat samples contained identical prey composition using genetic vs. hard-part methods of diet analysis. Whilst our ability to extract and sequence DNA has improved since 2009, the biological constraints that separate these two methods – ingestion or avoidance, and subsequent digestion or regurgitation of such tissues – likely have not.
* Lastly, the reviewer questions why we did not also attempt to extract DNA from hard-parts. Depending on the study objective, we agree that it is possible to combine morphological and genetic techniques in this way and by extracting DNA from specific pieces of non-identifiable prey tissues found in predator stomachs or hard-parts remaining in scats. However, this would represent a third and only partial assaying technique and is typically used to augment hard-part analyses. The usefulness of this approach must be balanced with the increased costs and time involved. In this proof-of-concept study, it was determined that it was more informative to assay simultaneously all samples with both a bulk morphological and DNA-based approach as these would be more comparable to past assays that only used one and no combination of these techniques for the study species and prey taxa. This may indeed be an area for future investigation. However, we do not include this in our discussion as we believe this to be outside the scope of the paper and possibly the body of work for a thesis. We are aware of a handful of papers that have gone to the trouble of extracting DNA from hard parts identified within scat samples, and this is typically done for smaller sample sizes.

Concern #2, regarding differentiation between species identified in samples and for haplotypes:

* In addition to providing the requested clarification (Methods, lines 171–181), we want to highlight that we also increased the stringency of our criteria for including a sequence in haplotype polymorphism analysis, to rule out intrusion of error beyond reasonable doubt. Our previous threshold for haplotype polymorphism analysis had been 1% of the total sequence abundance in each sample and included 12 sequences. We increased that threshold to ~7.5% of the total sequence abundance in each sample. We therefore include only 9 of the most abundant sequences and these came from only the 6 faecal samples that contained abundant DNA. As noted in our letter, this step therefore excluded over a thousand sequences and that follows our typical procedures for quality control and sequence filtering that already excluded 20,000+ sequences.
* Thus, we have thoroughly edited and clarified text in (Methods, lines 171–181): “We sought to identify a minimum number of individual little penguin by exploring mtDNA haplotypes from 12S rRNA sequences obtained. While dependent on sequence fidelity, such approaches have been used to explore intraspecific diversity (similar to Seersholm et al., 2018). We imported the quality-filtered file containing 47,478 seabird DNA sequences, produced just prior to OTU clustering, we disaggregated and matched these sequences in relation to sample identifier and formed these into clusters of unique sequences in Geneious. From the six samples that contained abundant penguin DNA (Table S4), we selected the nine most abundant unique sequences. Each of these represents a sequence abundance of greater than 7.5% of the total sequence abundance of the sample (Table S5). This process was used to exclude beyond reasonable doubt, any further sequences that could be attributed to sequencing error.”
* We also include a table (Table S5 in supplementary information) to be very clear on the abundance information for unique sequences selected for haplotype analysis.

Concern #3, regarding differentiation between species identified in samples and for haplotypes

* We trust that several of the points raised in this concern have been addressed in response to the first and second concerns, regarding conservative choices made at every step of the sampling and data processing steps of our genetic and morphological workflows. We hope all readers can now be confident that the haplotypes selected do not represent sequencing platform or other error, beyond reasonable doubt.
* Also, a great suggestion by the reviewer to double check any reference materials. Indeed, we added text (Results, lines 262–264): “Haplotype 1 was identified three different single sources of DNA from penguins from southeastern Australia (see reference material in Appendix S1.4).” The sources were one individual penguin carcass from Philip Island, Victoria, southern Australia, sampled at the time of this study, as well as two other whole genomes which we cite in Appendix S1.4 in our supplement (lines 129–132): “and matched the 12s rRNA gene of two independent penguin samples (MF370525, MK761006) from whole genomes from the same region (Sarker et al., 2017; Vianna et al., 2020).”
* All other sequences on GenBank come from New Zealand and likely belong to the other subspecies and not relevant to check for similar haplotypes for our study. We note that Tsuji et al. highlighted that the availability of vouchered reference haplotypes for many ecosystems and study species is still rare, yet they published over 900 haplotypes identified from wild eDNA samples, most of which had not yet been identified from single source material.
* Lastly, on the recommendation by the reviewer to extract and sequence DNA from “the penguin remains in fecal samples to generate haplotype sequences and frequency distribution and compare the data with fecal DNA-derived penguin haplotype diversity.” We do not feel that this is necessarily the best approach at this time and should be the subject of system-specific validation and testing in a captive feeding trial, for the reasons we outline in our response to the reviewer’s first comment. From our experience with ancient and degraded sources of DNA, we feel this would be risky as the DNA-poor hard parts would have been bathed in more DNA-rich soft tissues and stomach acids for hours and potentially contaminated prior to DNA extraction. Cleaning solutions like bleach may clean the outside of a bone or feather, but how effective this is throughout the matrix of a digested bone or feather is questionable and typically not desired. This isn’t a problem for other studies using DNA from hard-parts or additional soft tissues to augment morphological identifications, as they would only be seeking a species-level identification, but this is a problematic suggestion for haplotype polymorphism analysis. We posit that while interesting, this idea is outside the scope of this paper, and we sincerely hope that our extensive clarifications and significant editing of the original manuscript have provided much needed information and confidence in our work.

Again, we sincerely thank our anonymous reviewer for these salient comments and feedback. The manuscript is immeasurably stronger and more impactful now.