12-Aug-2021  
  
Dear Dr. Hardy,  
  
Thank you for submitting your manuscript "Quantifying wildlife conflicts by combining eDNA metabarcoding and traditional diet analysis" (21-372) to Conservation Biology. I have received a thorough, constructive review. The review is pasted below.  
  
Normally, we require 2 or 3 reviews. However, the recruitment of reviewers at this time of year has been slow. The review in hand is thoughtful and recommends 'reject-and-resubmit', raising significant concerns about the methods. Rather than delay things further, on the basis of the review and recommendation, I will decline the manuscript at this time, to give you time to consider these comments and, potentially, to revise accordingly. I will consider a resubmission that fully addresses the concerns described below.   
  
Resubmitting your manuscript does not guarantee eventual acceptance, and your resubmission will be subject to the full peer-review process. You have 90 days from the date of this letter to resubmit. If you anticipate resubmitting beyond that date, please contact me.  
  
To resubmit the manuscript, log into your Author Center at <https://mc.manuscriptcentral.com/conbio>. Click on "Manuscripts with Decisions" and then "Create a Resubmission," which is located next to the manuscript number. Then, follow the steps indicated. In the space provided or as a separate Word document, please include a detailed, point-by-point response to the comments of the handling editor and reviewers. Describe the changes you made to the original manuscript and, if applicable, explain why you did not address certain comments. If you have a Cover Letter, upload it as a separate document.  
  
Thank you.  
  
Sincerely,  
Mark Burgman  
Conservation Biology

REVIEWER COMMENTS  
  
Reviewer: 1  
Comments to the Author  
  
Interactions and conflicts between protected/valued species are interesting research topics and have implications for conservation planning. This study provides empirical evidence for predation between two such species, and the use of fecal DNA for quantitative estimation of the predation impact is relatively novel. However, I have major concerns about the technical treatments in this study (see detailed comments below), which should be carefully addressed for the results and conclusions to be credible.    
  
1. Although both morphological analysis and molecular diet analysis showed similar detection rates for seabirds, they detected seabirds in largely different samples (only 1/2–1/3 overlap). This low consistency between the results is alarming. For samples with visible prey parts (very good DNA source), it is baffling that the DNA analysis failed to pick the signal up. There could be important improvement to the DNA workflow to increase the detection sensitivity hence its reliability. For example, were the fecal samples thoroughly mixed and subsampled from multiple locations per pellet? Multiple independent PCR replicates are also necessary to increase detection probabilities. It was mentioned that duplicate PCRs were conducted, but I saw no further report in data processing and the results regarding the duplicates.  
  
2. During dietary sequence processing, sequences were clustered based on >97% similarity (L.180). One would expect haplotypes with <3% differences (= 7 nucleotides for a ~230 bp fragment) from each other to be collapsed into one sequence following this step. It would then be impossible to analyze haplotype diversity of the penguins in the samples. However, Fig. 5 showed 7 haplotypes, 6 of which (haplotypes 1-6) had < 5 nucleotides differences and should have been collapsed into one during sequencing processing. This is quite confusing.  
  
3. Another issue with haplotype assignments. Were all haplotypes recovered from fecal DNA also found in tissue samples from the local penguin populations, i.e., were they all true haplotypes or possibly errors generated during PCR and Illumina sequencing? Artificial errors are common in DNA metabarcoding, and there should be measures to specifically identify and eliminate them to ensure data credibility. Multiple PCR replicates, sequence filtering procedures, and statistical methods can all help to reduce errors (see Tsuji et al., 2020 Mol Ecol Resour 20:1248-1258 and Tsuji et al., 2020 Environ DNA 2:42-52). It would also be greatly helpful to use the penguin remains in fecal samples to generate haplotype sequences and frequency distribution, and compare the data with fecal DNA-derived penguin haplotype diversity.

RESUBMIT BY DECEMBER 10th!!!!

RESPONSE TO EDITOR & REVIEWER

We sincerely thank our anonymous reviewer and Prof. Mark Burgman, editor, for reviewing and 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 noticed that the concerns raised by the reviewer typically reflected a need for further or where information was lacking in the original submission. We addressed this throughout the manuscript with additional procedural information and further clarity in decisions taken and methods used. We thank the reviewer for this feedback, as this served to ensure that our manuscript is understandable and transferable to the wide readership of Conservation Biology.

We wish to highlight that we were conservative in all our sampling, DNA processing, sequencing and bioinformatics protocols. Indeed, we used conservative thresholds for inclusion of DNA at every step of the way, and we further exclude any sample that made it through all these steps and yet contained less than 2 sequences altogether, even though they present potentially real penguin consumption (Deagle et al. 2013). We then apply a polymorphism approach to a novel question and system, we tightened previously conservative thresholds for sequence selection and only selected the most abundant sequences (n = 9) within samples that contained abundant little penguin DNA (n = 6) and with a sequence abundance threshold for inclusion far higher (~7.5% of the total for each sample) than would be possible by platform error (1%). Indeed, our previous threshold for haplotype polymorphism analysis had been 1% of the total for each sample. We therefore exclude over a thousand sequences that could have resulted from potential sequencer error and above the already 30,000+ sequences that were removed in our stringent sequence filtering procedure. We provide further detail in this response and in our manuscript regarding protocols followed.

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 of the offshore island little penguin populations, 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

RESPONSE TO SPECIFIC CONCERNS:

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

The reviewer brings up several key details that needed further clarification and we document here how we address the reviewer’s first concern in the body of the manuscript below.

* Firstly, we initially used language such as “comparing” or “combining” methods, and to a certain degree we do because we are aiming to publish the results of both methods together (i.e., Deagle et al. 2009 who use different sets of scat samples and compared the overall diet both techniques used here). However, this language may have caused confusion, as we ultimately posit that these assay methods represent two nearly independent environmental surveillance and assaying techniques, and we recommend that they be applied simultaneously and for bulk sample processing similar to the work of Deagle et al. (2009). We have endeavoured to make this point and recommendation clear throughout the manuscript.

* We changed some language to use “apply” and we clarify the scope being “overall” as opposed to sample-by-sample (Abstract line 12, Introduction lines 114 and 123). We add the following text to guide readers expectations’ of our study and assaying techniques (Introduction lines …): “Due to known biological and methodological differences in dietary information obtained from hard-parts compared to soft tissues (Casper et al., 2007a, 2007b; Tollit et al., 2009), and differences in the quantities of DNA available from hard parts compared to soft tissues (McDonald & Griffith, 2011, Rothe & Nagy, 2016), we apply and present the results of the morphological and DNA metabarcoding techniques as simultaneously informative and complementary assaying techniques. Thus, 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 143–145 “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 air-tight, zip-lock bags.”
* Samples were indeed screened in duplicate using diagnostic endpoint PCRs which we noted formerly in lines 162–63, however we were too brief in describing what happened afterwards. We have added some clarifying text in Methods line 190 and the following detail from line 190–196: “A total of 32 samples showed target amplicons in both or one duplicate, whilst none of the extraction blanks and PCR blanks (negative controls) tested positive for target amplicons. We proceeded with sequencing using a single sample of DNA extract (neat concentration) for each of the 32 samples that tested positive for birds, and we included two extraction blanks and one positive control.”
* To clarify on sequence quality control and filtering, a concern of the reviewers both in their first and third issues raised, we heavily clarified and edited text in Methods lines 203–207: “…to process the paired-end sequences and retain only those sequences with exact matched genetic tags and primers for each sample. Primers and tags were removed after this initial filtering step to leave the target sequences. These target sequences were quality controlled and clustered into molecular operational taxonomic units (OTUs) using the *UPARSE* algorithm and a custom bioinformatics pipeline primarily performed in *USEARCH* (Edgar, 2010; Edgar & Flyvbjerg, 2015). Firstly, low abundance sequences (below a threshold of 1% of the total abundance of unique sequences) were removed to mitigate sequencing platform error and chimeras, and secondly sequences were 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 through standard sequence filtering and OTU clustering pipeline (with cluster size threshold value of 73), resulting in 47,478 filtered sequences across all 99 samples and 5 clustered OTUs.”
* We respectfully disagree with the reviewer’s comment that hard-parts are a ‘good source of DNA’, this would be a gross oversimplification of a complex body of knowledge and certainly we have not had the good fortune to easily extract DNA from such tissues (apparently nor have many others, McDonald & Griffith 2011 for example). 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 …).
* Thus to clarify how our work is seated within that context, we have added the following paragraph (Discussion, lines …): “While both methods provided statistically similar results, we emphasize that these represent quasi-independent assays of predation, complementary but not additive. Seabirds hard-parts occurred in more samples (n = 29), than their DNA (n = 21), and both types of remains for the same prey taxa were detected simultaneously by both methods in only 10 samples. Different detection rates between these methods are common across a broad range of predator and prey taxa (Tollit et al., 2009; Zarzoso-Lacoste et al., 2013; Mumma et al., 2016). Many methodological and biological factors differentially affect the recovery of diagnostic genetic and morphological materials. In another pinniped species, prey soft tissues may have a gut passage rate of 48 hours compared 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 scarcer DNA available from hard-parts, (i.e., feather, fur or bone). DNA from these chitinised or keratinized tissues is of poor quality and requires a different process for extraction (McDonald & Griffith, 2011, Zarzoso-Lacoste et al., 2013; 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.
* Lastly, the reviewer questions why we did not also attempt to extract DNA from hard-parts. Depending on the study objective, it is possible to formally 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. 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 is outside the scope of this paper and possibly the body of work for a thesis. We are only 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 and to augment morphological assays, which we do not recommend (lines …).

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

* We have thoroughly edited and clarified text in Methods (lines …): “Due to their high cultural and conservation value, we identify individual little penguin mtDNA haplotypes from the (12S rRNA) sequences obtained to estimate the minimum number of penguins consumed within samples assayed (similar to Seersholm et al., 2018). We imported the file containing 47,478 filtered seabird DNA sequences, 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 S5), 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 S6). This process was used to exclude sequences that could be attributed to sequencing error (Illumina’s Miseq typically results in a pattern of error that shows sequences with a single base pair change at an abundance of around 1% of the parent sequence; Berry, T. E., unpublished data).”
* We also include a table for sequence abundance information of the unique sequences selected for haplotype analysis….

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

* We believe this concern may have originated from confusion and lack of confidence in our methods, which we have thoroughly addressed in our responses to concerns #1 and #2 above, regarding conservative choices made at every step of the sampling and data processing steps of our genetic and morphological workflows.
* As outlined above, we are reasonably confident that the haplotypes selected do not represent sequencing platform or other error. We did sample and sequence the 12s gene for one individual penguin carcass from Philip Island, Victoria, southern Australia, and this haplotype was also identified in many samples from that region. The same haplotype matches an unverified sample on GenBank (MK761006) from an unknown source. 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.
* Check paper 🡪 one of the papers this reviewer highlights actually says that what they are requesting is not commonly possible at this time. Data on haplotypes is not commonly available for all study species.
* Lastly, the concern by the reviewer that “It would also be greatly helpful to use 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 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 being able to extract their DNA. 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. 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 the much needed information and confidence in our work.

**Cited herein – DO WE NEED THIS?**

McDonald, P. G. & Griffith, S. C. (2011). To pluck or not to pluck: the hidden ethical and scientific costs of relying on feathers as a primary source of DNA. Journal of Avian Biology **42**:3, 197-203. <https://doi.org/10.1111/j.1600-048X.2011.05365.x>