From cover letter:

Our paper demonstrates a reproducible protocol to identify target species from predator scat and, further, uses haplotype analyses to assess genetic diversity and probable abundances of prey species within and between scat samples. This novel research provides a powerful tool for quantifying predator-prey relationships. Our study advanced frameworks for processing DNA metabarcoding that enables us to determine the number of individual penguins consumed from scat samples collected from fur seal colonies across multiple sites. In conclusion, seabirds, particularly little penguins, are an important foraging strategy for some long-nosed fur seals. This research provides a critical step towards an up-to-date cumulative impact assessment for threats to little penguins in southern Australia.

Extra text from recent submission:

Additionally, increasingly frequent terrestrial heat waves can cause hyperthermia, whereby little penguins die of exposure to prolonged high ambient temperatures during the critical moult period (Lauren Tworkowski, La Trobe University, unpublished data). Additionally, a major driver of global penguin population vulnerability and decline involves food web changes caused by ocean warming and competition with marine fisheries (Ropert-Coudert et al., 2019).

We demonstrate significant advantages from DNA-based methods compared to morphological diet analysis for wildlife conflict surveillance between species of conservation priority.

Methods:

Long-nosed fur seals have only recently begun breeding in Bass Strait and NSW.

All samples containing little penguin DNA (n = 10) were then searched for the presence of haplotypes identified, to report on the 12S rRNA mitochondrial genetic diversity consumed by long-nosed fur seals, within and across samples.

Logically, two distinct mtDNA haplotypes (12s rRNA) found within a sample correspond to two distinct birds consumed.

Discussion:

* Towards significant advances in understanding complex predator-prey dynamics both within our local context and for the broader conservation biology community.
* Results can be related to previous research and assist in transitioning to more modern ecosystem surveillance methods.
* In our study system, this analysis provided key information to conservation practitioners for assessing an emerging wildlife conflict in Australian waters and to determine the next steps in monitoring and managing this conflict.
* Using the little penguin and long-nosed fur seal predator-prey model, we provide (i) a multi-assay method for comparison of target species identification to produce a more reliable prevalence than that offered by the traditional assay alone; (ii) a reproducible protocol for DNA metabarcoding analyses for identifying target prey species from predator scat samples; and, (iii) an applied haplotype polymorphism analysis for genetic diversity and probable abundances of target species within and between samples using shorter base-pair target DNA. Our analytical framework is reproducible and can be tailored to a broad range of wildlife interaction surveillance efforts.
* When diagnostic hard-parts are consumed, practitioners typically assign one individual to remains such as a pair of fish otoliths, a bird skull, paired wings or feet and paired upper with lower cephalopod beaks. However, assigning the number of individuals to remains such as feathers or fur has been simplistic and could over-represent predation by, for example, counting the presence of feathers in a single scat as one bird. To better quantify seabird predation, a controlled feeding trial identified that remains of a single penguin could appear in up to five separate fur seal scats on average (Goldsworthy et al. 2019). Additionally, recent scat clearing and re-sampling experiments indicated that penguin feathers, present in fur seal scats, may persist in the environment longer than finer particles (e.g., fish otoliths) (S-L Reinhold, unpublished data), likely adding to the overestimation of penguins in diet analyses when using morphological remains from old and fresh scats
* [Note that stringency likely eliminates legitimate genetic diversity, whilst account for sequencing error]. Therefore, the estimated number of penguins eaten (21 individuals) was higher than the prevalence of scats with penguin DNA (10 scats), so assuming that each scat with feathers represents one bird is simplistic and possibly an under-estimate not an over-estimate. Using haplotype detection, a single scat could contain up to six haplotypes or individual penguins.
* Additionally, Targeting longer and more variable barcodes would likely reveal greater genetic diversity and thus increase our estimation of individual penguins consumed, but at the loss of detecting shorter DNA traces typical in degraded faecal material (Taberlet et al. 2012).
* The likelihood for soft and hard prey remains to co-occur within the same samples.
* Quantifying predation can be difficult for certain taxa and current DNA-based tools already offer known and significant advantages over identifications of morphological prey remains, particularly for cartilaginous or gelatinous taxa. DNA analyses can detect larger prey missed in hard-parts analyses: for example, fur seals may break apart and selectively eat larger prey, remove seabird skin and feathers prior to consumption .and regurgitate large prey remains (Hocking et al., 2016; Page et al. 2005; Mumma et al., 2016). terrestrial have been reported to 2016). Morphological analyses of fur seal faeces can be problematic as they are Fur seals also engage in ‘belly-biting’ behaviours where soft parts of prey are targeted to the exclusion of diagnostic remains (Tollit et al., 2009; Granquist et al., 2018).
* followed. Wand . S). CThereIt is noteworthy that seabird DNA was amplified in up to 32 samples and sequenced successfully in 25 samples when we included samples with trace amounts of DNA. We chose to follow stringent sequence quality filtering to exclude the possibility of false positives beyond reasonable doubt, even though we did not obtain seabird DNA in our extraction blanks or negative controls, thereby making a false positive unlikely. Deagle et al. (2013) acknowledged that such stringent DNA processing steps would exclude true positives as well as quantitative information on prey consumption. Indeed, several fur seal samples contained trace amounts of DNA and morphological remains of the same taxon (ADD SI DATA), so our simultaneous detection rates of hard parts and DNA is conservative. Additionally, there may be some stochasticity in DNA amplification from a sample (Egeter et al., 2015). We mitigated this by thoroughly homogenizing samples and screening multiple replicates from the same sample, as well as using individually tagged PCR replicates. In future research, genetic methodologies could eclipse hard-parts analyses if genetic techniques can be used to augment morphological identifications by targeting non-identifiable prey tissues (Ford et al., 2011; Méheust et al., 2015).
* DNA metabarcoding provided key additional information here, critical to assessing predator-prey interactions within a wildlife conflict and conservation management context: (i) offering multiple metrics in addition to occurrence rates; (ii) detecting multiple prey taxa within a single sample; and (iii) identifying genetic diversity to estimate the abundances of consumed prey. We recommend the development and optimization of cost-effective assays tailored to the needs of specific wildlife conflict scenarios to better quantify and monitor these interactions. The use of multiple target genes typically produces more reliable results for determining predation prevalence and likely impacts. This method may be extended by including genetic screening to identify individual predators (Wegge et al., 2012), which would be valuable when considering controversial control strategies. If consumed biomass information is needed, we recommend developing DNA-to-tissue-based correction factors (Thomas et al., 2014). Numerous studies have developed species-specific and cost-effective assays using older technology, which could be applied to large sample sizes and large numbers of predatory taxa for the detection of specific taxonomic groups of high conservation or commercial interest (Fox et al., 2012; Hunter et al., 2012; Schreier et al., 2016). For example, Skaala et al. (2014) used genetic techniques not only to identify the prey species of interest, but also used several microsatellite markers to identify the origin of prey stock at high spatial resolution.

References

* Deagle et al. (2009)
* Fox et al. (2012) \_ADDED BACK
* Hunter, E., Taylor, N., Fox, C. J., Maillard, M., & Taylor, M. I. (2012). Effectiveness of TaqMan probes for detection of fish eggs and larvae in the stomach contents of a teleost predator. Journal of Fish Biology **81**:1, 320–328. <https://doi.org/10.1111/j.1095-8649.2012.03298.x>
* Schreier, B. M., Baerwald, M. R., Conrad, J. L., Schumer, G., & May, B. (2016). Examination of Predation on Early Life Stage Delta Smelt in the San Francisco Estuary Using DNA Diet Analysis. Transactions of the American Fisheries Society **145**:4, 723–733. <https://doi.org/10.1080/00028487.2016.1152299>
* Kirkwood et al. (2008)
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* Skaala et al. (2014)
* Taberlet, P., et al. (2012). "Towards next-generation biodiversity assessment using DNA metabarcoding." Molecular Ecology **21**:8, 2045–2050.

Conclusions

This study uses a valuable multi-assay framework for identifying and quantifying predation, that can be broadly applied across systems and ecological issues. Here we identify the prevalence of seabird predation and estimate the abundance of little penguin predation by long-nosed fur seals, information that is critical to assessing and managing an emerging wildlife conflict in Australian waters. We identify the genetic remains of between 1–6 individual penguin haplotypes within each long-nosed fur seal scat sample that tested positive for penguins and represent a minimum of 21 little penguins consumed across 99 predator samples. DNA-based diet analysis also identified the remains of multiple seabird taxa within some samples, indicating seabird predation may be a relatively important individual foraging strategy for some fur seals. Using both morphological and genetic diet assays, we estimate an overall range in predation detection of 9–29% for seabirds, and 6–25% for little penguins. These detection rates warrant further monitoring and quantitative investigation through longer-term and more comprehensive sampling programs across southern Australia to provide managers with robust spatio-temporal predation patterns and estimates. Finally, with ongoing genetic technological advancement, we recommend the development of cost-effective assays tailored to the needs of specific wildlife conflict scenarios in order to better quantify these conflicts.

Intro:

Little penguins and other seabirds have been identified in the diets of juveniles, sub-adult and adult male long-nosed fur seals, at two locations in southern Australia: (i) from little penguin DNA in a single scat at a site at the northeastern edge of the range (Hardy et al., 2017); and, (ii) from a 3-year scat sampling program at a large, southern breeding colony where little penguin morphological remains were detected in up to 5.9% of samples, and shearwaters in up to 1.3% of those samples (Page et al., 2005).

Discussion

We identified statistically similar rates of occurrence for seabirds and little penguins from both the genetic and traditional assay methods. However, DNA metabarcoding provided key additional information critical to assessing predator-prey interactions within a wildlife conflict and conservation management context: (i) a prevalence range based on recovered genetic abundance and that is likely to be more reliable than traditional morphological assays; (ii) detection of multiple prey taxa within a single sample; and (iii) identification of genetic diversity enabling estimation of penguin abundances consumed. DNA metabarcoding also offers multiple metrics in addition to occurrence rates and helped to form a consensus here that little penguins are currently the most commonly consumed seabird by long-nosed fur seals in comparison to other seabirds (e.g., procellarids, black-browed albatross, greater crested tern, and Australasian gannet).

Previously, a single fur seal scat containing feathers regardless of amount has been hypothesised to represent one bird (Page et al., 2005).

Future studies could screen for predator genetic diversity to identify individuals in a population contributing to predation of a sensitive or valuable species (Wegge et al., 2012), particularly when considering controversial predator population control strategies. Species-specific probes could be developed for penguins using older and cheaper genomic technology (Fox et al., 2012), cross-validation of eDNA data with quantitative PCR (Murray et al. 2011), or development of penguin-specific DNA-to-tissue-based correction factors could provide consumed biomass information (Thomas et al., 2014).

Prey not consumed whole / prey processing:

Hocking, D. P., Fitzgerald, E. M. G., Salverson, M., & Evans, A. R. (2016). Prey capture and processing behaviors vary with prey size and shape in Australian and subantarctic fur seals. Marine Mammal Science **32**:2, 568–587. [doi.org/10.1111/mms.12285](https://doi.org/10.1111/mms.12285)

Supplement:

The success of this colony is largely attributed to fox eradication and habitat restoration [(Rout et al., 2014; Sutherland & Dann, 2014)](https://www.zotero.org/google-docs/?XUwBYO). The internationally popular Penguin Parade, managed by Phillip Island Nature Parks (The Nature Parks), provided little penguin viewing experiences for over 757,846 visitors in 2019-20 [(Phillip Island Nature Parks, 2020)](https://www.zotero.org/google-docs/?qItaWv).

[Phillip Island Nature Parks. (2020).](https://www.zotero.org/google-docs/?KBzpyp) *[Annual Report 2019-20](https://www.zotero.org/google-docs/?KBzpyp)* [(ISSN 2201-2842). www.penguins.org.au/assets/About/PDF-Publications/Nature-Parks-Annual-Report-2019-20.pdf](https://www.zotero.org/google-docs/?KBzpyp)

[Rout, T. M., Kirkwood, R., Sutherland, D. R., Murphy, S., & McCarthy, M. A. (2014). When to declare successful eradication of an invasive predator? *Animal Conservation*, *17*(2), 125–132. doi.org/10.1111/acv.12065](https://www.zotero.org/google-docs/?KBzpyp)