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Estimating diet of the European hedgehog (*Erinaceus europaeus*) using molecular analysis of faeces and colon content.

A thesis
submitted in partial fulfilment
of the requirements for the Degree of
Master of Science

at
Lincoln University
by
Maryanne Rachel Walker

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by

Maryanne Rachel Walker

New Zealand has a unique ecosystem that evolved without the presence of mammalian predators. Since human colonisation, several species of mammalian predators have been introduced, including the European hedgehog (*Erinaceus europaeus*). The hedgehog, in its native range, mainly feeds on invertebrates, with minor dietary components of bird eggs and lizards. I used molecular methods to analyse the diet of hedgehog in New Zealand. Molecular methods have an important advantage over the traditional visual analysis of faeces and stomach contents as they allow for better identification of soft-bodied remnants of prey items. I successfully used species-specific primers to detect a known species (mealworms, *Tenebrio molitor*) in hedgehog faeces after a controlled feeding trial.

Next, I trialled a simple visualisation method to compare the quality of DNA extracted from hedgehog stomach and colon contents. Unfortunately, the visualisation method trialled did not determine any differences in the DNA quality of the two sample sources and further trials are required. I created a local reference database of invertebrates collected from pitfall trapping at Kaitorete Spit. Barcode sequences developed for the local reference library were uploaded to BOLD systems to contribute to the growing global database of sequences. This local reference database successfully enhanced the taxonomic rank assignment of amplicon sequence variations produced during metabarcoding. I used high throughput sequencing on faecal and colon samples. Several taxa from the phyla Arthropoda, Annelida, Mollusca, Nematoda, and Rotifera were identified and reported at the genus and family (for Arthropoda) taxonomic level. There was a considerable level of taxon heterogeneity in the prey items not between samples. High abundances of Arthropoda were identified in colon samples, while faecal samples had higher abundances of all other phyla. The results from this study are consistent with earlier morphological studies of hedgehog diets in New Zealand and Europe but add greater detail. The dietary results from this study are important and applicable in both New Zealand and worldwide. For New Zealand, this study contributes to understanding the ecological impacts that hedgehogs have in New Zealand while providing justification for ongoing monitoring plans that

control hedgehogs to prevent detrimental impacts. Worldwide, these methods can be used, especially in the hedgehog's natural habitat, to understand their natural diet to assist with conservation where this species is endangered. Future studies should expand the area studied to explore dietary variation across a spatial scale and where other endangered species are at risk from hedgehog predation.

Keywords: COI, diet, environmental DNA, Erinaceus europaeus, European hedgehog, high throughput sequencing, New Zealand, species-specific primers

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Table of Contents

Abstract .... **iii**

Acknowledgements .... **v**

Table of Contents .... **vi**

List of Tables .... **ix**

List of Figures .... **x**

Chapter 1 Introduction **1**

1.1 The European hedgehog 2

1.2 Ecology 2

1.3 The introduction of hedgehogs into New Zealand 3

1.4 Hedgehog diet in Europe 4

1.5 Hedgehog diet in New Zealand 5

1.6 Control methods in New Zealand 5

1.7 DNA methods used for diet analysis. 6

1.8 Research Intent 9

1.9 Study Site 11

1.10 References 13

Chapter 2 Developing a DNA method to detect a known dietary item (mealworms, *Tenebrio molitor* larvae) in hedgehog (*Erinaceus europaeus*) faeces. **19**

2.1 Introduction 19

2.2 Objectives 21

2.2.1 Undertake a trial feeding mealworms to hedgehogs in a controlled environment. 21

2.2.2 Design primers to detect mealworms from raw mealworm DNA. 21

2.2.3 Determine PCR parameters to detect mealworms in hedgehog feaces. 21

2.3 Methods 21

2.3.1 Mealworm Feeding Trial 21

2.3.2 Primer Design 22

2.3.3 DNA Analysis of faecal content 23

2.3.4 Sequencing Mealworm DNA 24

2.4 Results 25

2.4.1 Mealworm Trial Feeding Rates 25

2.4.2 Primer Design 25

2.4.3 Non-Target Primer Testing 26

2.4.4 Faecal analysis 27

Sequence PCR output 28

2.5 Discussion 28

2.6 Conclusion 30

References 31

Chapter 3 Comparing the quality of DNA in hedgehog's (*Erinaceus europaeus*) stomach and colon. **34**

3.1 Introduction 34

3.2 Objective 35

3.2.1 Compare the DNA output in the stomach and colon content. 35
3.3 Methods 36
3.3.1 Collection of Hedgehogs 36
3.3.2 Dissection and collection of stomach and colon content 36
3.3.3 DNA analysis. 36
3.4 Results 37
3.5 Discussion. 38
3.6 Conclusion 39
3.7 References 40

Chapter 4 A DNA reference library of invertebrates from Kaitorete Spit. 43
4.1 Introduction 43
4.2 Objective 44
4.3 Methods 45
4.3.1 Study Site 45
4.3.2 Collection of Species 45
4.3.3 Curation and Identification 46
4.3.4 Barcoding DNA 46
4.3.5 BOLD systems 47
4.4 Results 49
4.5 Discussion. 55
4.6 Conclusion 56
References 57

Chapter 5 European hedgehog (*Erinaceus europaeus*) diet in New Zealand determined using high throughput sequencing of faecal DNA. 60
5.1 Introduction 60
5.2 Objectives 62
5.2.1 Undertake metabarcoding analysis of hedgehog faecal and colon content to determine the diet of hedgehogs on Kaitorete Spit. 62
5.2.2 Evaluate the taxonomic assignment of sequences from the locally generated reference database of sequences (see Chapter 4) to sequences generated through metabarcoding to identify site-specific invertebrates that may not have had publicly databased sequenes available. 62
5.2.3 Analyse the potential for this methodology to be used as a complementary monitoring method to be integrated into the current predator control plans on Kaitorete Spit. 62
5.3 Methods 63
5.3.1 Faecal sample collection 63
5.3.2 Colon collection 63
5.3.3 DNA extraction 63
5.3.4 High Throughput Sequencing (HTS) 63
5.3.5 Bioinfortmatics Analysis 64
5.4 Results 67
5.4.1 Raw DNA visualization and data. 67
5.4.2 High Throughput Sequencing 68
5.4.3 Barcoded Specimens 68
5.4.4 Anacapa 70
5.5 Discussion. 79

5.5.1 The critical role of a local reference database 80
5.5.2 General comments 82
5.5.3 Limitations and future directions of the study 83
5.5.4 Conclusion 84
5.6 References 86

Chapter 6 Conclusion 91
References 93

Appendix A Detecting Mistletoe in Possum Scat 94
A.1 Introduction 94
A.2 Aims 96
A.3 Methods 97
A.4 Results 99
A.5 Discussion 101
References 103

List of Tables

Table 2-1. Mealworm feeding trial results. *Hedgehog 11 broke into Hedgehog 9's pen. 25
Table 2-2. Criteria for the specific primer design of the mealworm COI gene (*Tenebrio molitor*). ..25
Table 2-3. Lab code and species name of non-target species against the mealworm-specific primer. 26
Table 2-4. Diet design, weight, and proportion of faecal subsamples from the mealworm feeding trial. 27
Table 3-1. Sample weight and Nanodrop output from raw DNA samples of hedgehog stomach and colon content. 37
Table 4-1. Barcode of Life Database pages for all sequenced specimens. The specimen page includes collection information, photographs, and taxonomy (Figure 4-3). Morphological ID is from 4.3.3. The sequence page includes the illustrative barcode and nucleotide sequence (Figure 4-4). The BIN number collates all records of the same sequence into one page (Figure 4-5). Each species contains hyperlinks for the specimen page, sequence page and BIN number. 47
Table 4-2. The number of each invertebrate order that was sequenced for the invertebrate library. See Table 4-1 for the full identification list. 49
Table 4-3. List of the Invertebrate library comparing the morphological identification and DNA identification showing the percentage match of the DNA matches to public databases. Grey rows indicate unsuccessful DNA sequencing and a green to yellow colouration shows the percentage match with 100% being green. Of the sequences in the DNA library, 25 had grades >99% (14 species, six genera, and five family level). .50
Table 5-1. Nanodrop readings of quality, quantity, and raw sample weight. 67
Table 5-2. Output from Multi QC report for each sample. R1 = forward, R2 = reverse, M Seqs = Million Sequences. 68
Table 5-3. Summary of species matches from the invertebrate library (Chapter 4) in the high throughput sequencing of colon and faecal content. 69
Table 5-4. Species matches from HTS for faecal samples with the number of sequences and the percentage match to order and species identifications. 69
Table 5-5. Species matches from HTS for colon content samples with the number of sequences and the percentage match to the identification. 69
Table 5-6. Reference list of sample codes matching the collection code and the code after Anacapa analysis. 70
Table 5-7. Statistical test results for the observed diversity 75
Table 5-8. Statistical test results for the Shannon diversity 77
Table 5-9. Multivariable ANOVA table for the Jay-Curtis sample analysis......................................77
Table 5-10. Multivariable ANOVA table for the Jay-Curtis Source analysis 78

Table A. 1 Biomass data of the pen trials of possum mistletoe consumption................................99

List of Figures

Figure 1-1. European Hedgehog (*Erinaceus europaeus*) in exotic pasture in Rotorua, New Zealand (Sourced from M. R. Walker 2024) 2
Figure 1-2. Map of the South Island of New Zealand. Yellow arrow is Kaitorete Spit. Retrieved from Google Earth. 11
Figure 2-1. Pen used to hold hedgehogs during the mealworm feeding trial in February 2023. 22
Figure 2-2. Feeding dishes for the mealworm feeding trial with 10, one or no mealworms and the maintenance diet. 22
Figure 2-3. Mealworm-specific primer testing on mealworm (MW) DNA at 50 °C, 55 °C, and 60 °C. 25
Figure 2-4. Non-target primer testing gel 26
Figure 2-5. Non-Target primer testing gel electrophoresis at an annealing temperature of 60 °C, 62 °C, and 64 °C of the false positives. 26
Figure 2-6. Gel electrophoresis output of PCR of faeces from hedgehogs fed mealworms. H = Hedgehog, N = Night of feeding. *H5N3 was the only faecal sample with a positive detection. 27
Figure 3-1. Crude DNA comparing stomach and colon DNA on gel electrophoresis. 37
Figure 4-1. Map of Kaitorete Spit with Kaitorete Reserve in white. Retrieved from Google Earth. 45
Figure 4-2. The layout of pitfall traps at Kaitorete Reserve that was established by the Christchurch City Council. Retrieved from Google Earth. 46
Figure 4-3. Example of a BOLD specimen page for sample NZ750_2023 - *Pericoptus* sp. 52
Figure 4-4. Example BOLD sequence page for sample NZ750_2023 - *Pericoptus* sp. 53
Figure 4-5. Example BIN page from BOLD systems for sample NZ750_2023 - *Pericoptus* sp. 54
Figure 5-1. Gel electrophoresis from crude DNA was extracted and sent to Austria for high-throughput sequencing analysis. *Indicates samples that did not amplify in PCR. 67
Figure 5-2. Comparison of the number of ASVs (amplicon sequence variation) at the phylum taxonomic level and the sample source (hedgehog faecal and colon content) using rAnacapa. 71
Figure 5-3. Comparison of the proportion of Arthropoda families identified through HTS (high throughput sequencing) and the sample source (hedgehog faecal and colon content) using rAnacapa. 72
Figure 5-4. Comparison of the proportion of Arthropoda genera identified through HTS (high throughput sequencing) and the sample source (hedgehog faecal and colon content) using rAnacapa. 72
Figure 5-5. Comparison of the proportion of Annelida genera identified through HTS (high throughput sequencing) and the sample source (hedgehog faecal and colon content) using rAnacapa. 73
Figure 5-6. Comparison of the proportion of Mollusca genera identified through HTS (high throughput sequencing) and the sample source (hedgehog faecal and colon content) using rAnacapa. 73
Figure 5-7. Comparison of the proportion of Nematoda genera identified through HTS (high throughput sequencing) and the sample source (hedgehog faecal and colon content) using rAnacapa. 74
Figure 5-8. Comparison of the proportion of Rotifera genera identified through HTS (high throughput sequencing) and the sample source (hedgehog faecal and colon content) using rAnacapa. 74
Figure 5-9. Visualisation of the observed diversity for the taxonomic identifications in each sample analysed for the diet of hedgehogs. 75
Figure 5-10. The distribution of the observed diversity for the taxonomic identifications in each of the sample types analysed for the diet of hedgehogs. 75
Figure 5-11. Visualisation of the Shannon diversity for the taxonomic identifications in each sample analysed for the diet of hedgehogs. 76

Figure 5-12. The distribution of the Shannon diversity for the taxonomic identifications in each of the sample types analysed for the diet of hedgehogs. 76
Figure 5-13. Comparison of the Jaccard dissimilarity of the taxonomic identifications in the individual samples for the diet of hedgehogs. The PCoA plot shows 30.8% of the total variance between each sample. 77
Figure 5-14. Comparison of the Jaccard dissimilarity of the taxonomic identifications in each sample type for the diet of hedgehogs. The PCoA plot shows 30.8% of the total variance between the sample sources. 78

Figure A. 1 White Mistletoe pre feed (Left) and post feed (Right) (Sample 16). 99
Figure A. 2. PCR gel run of Possum Poo with Mistletoe Primer. The sample code and description are to the right of the image. 100

Chapter 1

Introduction

New Zealand has a unique endemic flora and fauna that has evolved without terrestrial predatory mammals, resulting in a ground-based ecosystem with slow breeding life histories. The first mammalian predator introduced by Polynesian settlers into New Zealand was the kiore, the Polynesian rat (*Rattus exulans* (Peale, 1848)) (Taylor 1975). The kiore became abundant in New Zealand before displacement by other rat species (*Rattus norvegicus* (Berkenhout, 1769) and *R. rattus* (Linnaeus, 1758)) introduced by European settlers (Taylor 1975). Further introduction of mammals by European settlers included mice (*Mus musculus* Linnaeus, 1758)) (Marris 2000), mustelids (stoats (*Mustela erminea* Linnaeus, 1758), ferrets (*M. furo* Linnaeus, 1758), and weasels (*M. nivalis* Linnaeus, 1758)) (Schlesselmann et al. 2018), possums (*Trichosurus vulpecula* (Kerr, 1792)) (Montague 2000) and hedgehogs (*Erinaceus europaeus* Linnaeus, 1758) (Fountain et al. 2013). The introduction of many of these mammalian species was intentional, to remind colonisers of home (e.g. hedgehogs (Bull 1968)), to provide a fur trade (e.g. possums (Pracy 1974)) or to control other introduced species (e.g. mustelids for rabbit control, *Oryctolagus cuniculus* (Linnaeus, 1758) (National Pest Control Agencies 2018)). Other introductions were accidental, due to animals arriving on ships or among imported goods (e.g. rodents (King 2023)).

1.1 The European hedgehog

Figure 1-1. European Hedgehog (*Erinaceus europaeus*) in exotic pasture in Rotorua, New Zealand (Sourced from M. R. Walker 2024)

The European hedgehog (hereafter hedgehog) is a small, oval-shaped mammal, about 22 cm long and weighing between 300–1300 g depending on the season (Figure 1-1) (King & Morris 2008). The hedgehog has a grey-brown back with 2–2.5 cm spines with yellow tips, black eyes, and round ears (King & Morris 2008). The underside and face of the hedgehog is covered with coarse grey-brown hair, and it has hairless black feet and a 2 cm hairless black tail (King & Morris 2008).

1.2 Ecology

The hedgehog naturally occurs in Europe, from Finland (Rautio et al. 2016) and Italy (Di Nicola et al. 2021), to the neighbouring island of Britain (Yalden 1976). Hedgehogs live in a range of habitats, including arable farms in Britain (Hof & Bright 2010) and urban areas of the United Kingdom (UK) (Gazzard et al. 2022). In arable farms, hedgerows and field margins provide important shelter, nesting sites, food (Hof & Bright 2010), and protection from the hedgehog's main predator, the European badger (*Meles meles* (Linnaeus, 1758)) (Young et al. 2006). The abundance of hedgehogs varies directly with the density of badgers and food resources (Micol et al. 1994). Gazzard et al. (2022), in an urban study, found that residential gardens are the preferred habitat of hedgehogs while regularly maintained grasslands with regular dog (*Canis familiaris* Linnaeus, 1758) visits were the least preferred. The high use of gardens shows the potential for gardens to be important in the conservation of hedgehogs in the UK (Gazzard et al. 2022). Nesting locations vary depending on the habitat and availability of refugia (Marco-Tresserras & López-Iborra 2023). In urban forests,

hedgehogs mainly nest under shrublike plants, such as *Salsola oppositifolia* Desf, and on a university campus, under sturdy plants with *Hedera helix* L. (Marco-Tresserras & López-Iborra 2023).

In New Zealand, hedgehogs inhabit most habitats, including urban areas (Brockie 1959), farmland (Campbell 1973), forests (King et al. 1996) and alpine zones (Foster et al. 2021). The ideal habitat for hedgehogs in New Zealand is lowland dairy pasture (King 2023) or areas with few frosts and many snails or gardens (King & Morris 2008). Hedgehogs have also been reported from braided rivers feeding on ground-nesting birds (Sanders & Maloney 2002). King et al. (1996) found that hedgehogs were common in unlogged native forests far from any roads and more so in older exotic forests.

Hedgehogs hibernate over-winter when food supply is low, and temperatures are too cold (<8 °C) (South et al. 2020). The frequency of hedgehogs seen hibernating in urban areas has increased, directly influenced by supplementary feeding from people during the summer (Gazzard & Baker 2020). Feeding throughout winter has unknown effects on the hibernation process as hedgehogs wake from hibernation to feed unnecessarily (Gazzard & Baker 2020). In a hedgehog rescue centre, weight loss over winter was related to the number of nights asleep, not arousal events (South et al. 2020), indicating that supplemental feeding may have a benefit in preventing weight loss; however, research is still needed in this area.

In New Zealand, few hedgehogs south of Taupo are seen during winter months due to hibernation (King et al. 1996). In the north of New Zealand, few hedgehogs hibernate due to the mean earth temperature remaining above 10 °C (King et al. 1996). Despite an aversion to cold climates, hedgehogs have been found to successfully rear young and hibernate in the alpine zones of New Zealand (Foster et al. 2021).

1.3 The introduction of hedgehogs into New Zealand

The hedgehog has become widespread in New Zealand after being introduced in the 1870s to control introduced garden pests, such as slugs and snails, and to remind European settlers of home (Bull 1968). The initial introduction started in Christchurch (South Island) in 1870 before later introductions in the North Island (Brockie 1959; Pipek 2020). The Canterbury Acclimatisation Society, responsible for the introduction of many non-native species, received two hedgehogs in 1870, and another the following year with an unknown fate (Bull 1968). In 1894, 12 hedgehogs were received by Mr P. Cunningham of Merivale (Canterbury), and subsequently escaped from captivity, possibly leading to the current population in Canterbury (Bull 1968; Pipek 2020). Further unrecorded liberations throughout Canterbury and New Zealand likely increased the distribution of hedgehogs

and by 1948 hedgehogs had spread throughout Canterbury, except in high mountain ranges (Bull 1968).

Hedgehogs were introduced in the North Island from shipments directly from Europe and relocations from Canterbury (Pipek 2020). Some hedgehogs in Auckland and Wellington appeared as pets without previous shipment records, indicating that the actual shipments occurred earlier than recorded (1890s) (Pipek 2020). Hedgehogs continued to spread and become a pest in many other parts of New Zealand, being abundant in urban areas (Brockie 1959) and farmland (Campbell 1973). More recent observations have been reported from native bush habitats (Berry 1999; King et al. 1996) and alpine zones where they have been seen hibernating, indicating hedgehogs have now established in alpine habitats (Foster et al. 2021).

1.4 Hedgehog diet in Europe

Hedgehogs, although considered omnivores, mainly feed on invertebrates (King & Morris 2008). In Europe and Britain, invertebrates dominate the hedgehog's diet (Rautio et al. 2016; Yalden 1976). Stomach analysis of Fenn-trapped hedgehogs in England showed that Coleoptera dominated the stomachs with remnants found in 75% of stomachs (60% Carabidae) (Yalden 1976). Other important dietary invertebrates were Dermaptera (58%) and Lepidoptera (49%) with small occurrences of both bird (12%) and egg (11%) fragments (Yalden 1976).

A dietary analysis in Finland, the hedgehog's northern distribution limit, found the same dominant dietary items in hedgehog stomachs of Coleoptera (80%), earthworms (50%) and caterpillars (45%), as well as a high abundance of human-related food (90%) (Rautio et al. 2016). Rautio et al. (2016) deemed the abundance of human food a benefit as it provides supplementary feeding in a suitable sheltered habitat. Morris (1985) also researched the impacts of human food on hedgehogs in a London golf course and a separate group of gardens. Hedgehogs did not become reliant on supplementary feeding of milk and bread in the urban feeding experiment (Morris 1985). Hedgehog movements remained unpredictable, despite regular food availability, normal foraging was not neglected, and no defence behaviour was observed (Morris 1985). The supplementary food was just that, a supplement, and not a replacement for the hedgehogs' normal diet routine (Morris 1985).

Dickman (1987) studied changes between juvenile and adult hedgehog diets in southern England. The diet changed from a dominance of Isopoda, Arachnida and Dermaptera as juveniles to larger prey of molluscs, larvae and Carabidae beetle as adults (Dickman 1987). The change in diet reveals that young individuals feed from the entire prey spectrum, while adults may specialise on a narrow range of prey (Dickman 1987). However, the small sample size of adults across three regions may

have caused a bias, and this conclusion should be used cautiously (Dickman 1987). Yalden (1976) also observed a change in the diet of young and adult hedgehogs with older individuals feeding more on earthworms, Carabid beetles, and slugs. Young hedgehogs may have preyed on vertebrate carrion more than older individuals due to being unable to find invertebrate food (Yalden 1976) supporting the hypothesis of Dickman (1987) that adults are, unsurprisingly, more skilled hunters than juveniles.

1.5 Hedgehog diet in New Zealand

The hedgehog diet in New Zealand reflects what has been observed in the dietary studies in Europe. In New Zealand, hedgehog stomach contents have high proportions of millipedes (80%), beetles (53%), earthworms (43%) and weta (Rhaphidophoroidea Walker, 1869) (13%) (Nottingham et al. 2019). In New Zealand irrigated pastures, earwigs and lepidopteran larvae were the most important invertebrates in a hedgehog's diet (Campbell 1973). During the grass-grub beetle (*Costelytra giveni* Coca-Abia & Romero-Samper, 2016) flight season, these beetles were the dominant food item in the hedgehog diet (Campbell 1973). In dryland pastures, Jones and Norbury (2011) found the most commonly eaten foods were beetles (94% of faeces), earwigs (92%), spiders (25%) and skinks (14%), which mirrored the food availability at the site. Hedgehogs cause a predation risk to most large invertebrates (Fountain et al. 2013) and at low frequencies feed on lizards, birds' eggs and ground-nesting chicks (Collins 2015; Jones et al. 2005; Nottingham et al. 2019). Although hedgehogs are not a major predator of birds and lizards, small-localised populations may be threatened by hedgehog predation (Jones et al. 2005).

Geographically restricted invertebrates are at significant risk from hedgehog predation (Fountain et al. 2013). The Canterbury knobbled weevil (*Hadramphus tuberculatus* (Pascoe, 1877)) is only known from one location in Canterbury, after being rediscovered in 2004 (Young et al. 2008) and has the potential to be devastated by hedgehog predation (Fountain et al. 2013). The predation pressure from hedgehogs decreases the abundance of the prey species and affects the wider food chain by creating competition for native insectivorous birds and invertebrates (Berry 1999). Berry (1999) determined that the diet of hedgehogs and North Island brown kiwi (*Apteryx mantelli* Bartlett, 1852) have a significant overlap, and, during autumn, hedgehogs may cause significant competition for food resources with kiwi when hedgehogs prepare for hibernation.

1.6 Control methods in New Zealand

In the 1930s, soon after the hedgehogs had become abundant in much of New Zealand, a bounty was placed on hedgehogs to motivate locals to assist with control efforts, because hedgehogs were

deemed a menace to ground-nesting birds (Brockie 1959). During the 1930s, 53,647 snouts were submitted in the North Island (Brockie 1959) and at least another 4,752 snouts were paid out by the Canterbury Acclimatization Society (Bull 1968).

Current-day control methods rely on trapping because hedgehogs have a reputation for toxin resistance (Berry 1999). These traps include the DOC 150, DOC 200, and DOC 250 traps (Department of Conservation n.d.) baited with peanut butter, rabbit meat, or chicken eggs (*Gallus gallus* (Linnaeus, 1758)) (Predator Free NZ n.d.). The entrance hole size of the DOC 250 traps (80 x 80 mm) allows entry for all but the largest of hedgehogs (>1.53 kg) and no hedgehogs in the study by Jones et al. (2021) (weight 0.85–1.53 kg) fit through the 60 x 60 mm entrance hole of the DOC 150 and 200 traps. Only targeting small hedgehogs could have serious implications for management plans where larger hedgehogs are more likely to survive winter and will not be controlled, or where the traps do not effectively kill the hedgehogs that are small enough to enter (Jones et al. 2021). The DOC 250 trap is the best current option on the market; however, there is a public demand for the standardisation of methods to control hedgehogs and better traps that do not exclude larger individuals (Jones et al. 2021). This lack of specific control methods emphasises the need to understand hedgehog ecology and ecological impacts. Further research on the detrimental effects of hedgehogs could add strength to the argument that New Zealand needs to control hedgehogs to a level alike rats and possums.

1.7 DNA methods used for diet analysis.

To date, research on the hedgehogs' diet has used methods of visual-hand sampling of stomach and faecal contents (Nottingham et al. 2019; Rautio et al. 2016). Although a widely used method of dietary analysis for a range of species, the visual-hand analysis method can underestimate the number of invertebrates in faeces due to limited hard body remnants in the sample (Dickman & Huang 1988) and soft body parts are often too small for visual identification using a microscope (Monterroso et al. 2019). An advantage of analysing faecal matter over stomach contents allows for dietary analysis studies without killing the target animals (Dickman & Huang 1988). The current control methods of trapping in New Zealand allow for stomach content analysis with minimal extra labour or animal killing because these animals are already killed during predator control programmes.

The historical method of visual-hand sampling has determined a baseline of invertebrates that hedgehogs predate on, with a small number of species identifications (Campbell 1973; Fountain et al. 2013). Molecular methods can provide additional identifications from small fragments of DNA

from soft body parts (Emami-Khoyi et al. 2016; Thomsen & Willerslev 2015). Without molecular methods, species identification of some dietary items, such as bird feathers and eggshells, is usually too difficult for identification beyond order level (Dowding et al. 2015).

The molecular methods of polymerase chain reaction (PCR) and high throughput sequencing (HTS) are used in this thesis. Polymerase chain reaction amplifies extracted DNA using primers designed to target a specific species or a taxon group (Gonçalves et al. 2014). Polymerase chain reactions can also be used to detect a target species in the diet of invertebrates and mammals (Bronnenhuber & Wilson 2013). Vink et al. (2011) tested species-specific primers for analysing the diet of predatory spiders in pasture environments. In the lab, *Anoteropsis hilaris* (L. Koch, 1877) spiders were fed a known invertebrate diet of *Sminthurus* sp. P.A. Latreille, 1802 (Vink et al. 2011). Species-specific primers were designed to target the prey species, but not the predator, and were successful at amplifying only the prey DNA (Vink et al. 2011). Further testing of non-target species would be needed before this process could be applied to the wild diet of predators collected from the field, after which, prey-specific primers could be used to determine if predators provide unknown biological control benefits or predate on other beneficial species (Vink et al. 2011).

Other studies have successfully designed specific primers for detecting rare fish in freshwater samples (Bronnenhuber & Wilson 2013) and the dietary items of captive stellar sea lions (*Eumetopias jubatus* (Schreber, 1776)) (Deagle et al. 2005). By using species specific primers, Vink and Kean (2013) determined that the spider *Tenuiphantes tenuis* (Blackwall 1852) is a significant predator of the Argentine stem weevil (*Listronotus bonariensis* (Kuschel, 1955)) and the spider could be used as a biological control organism. In a lab trial, hedgehog, rat (*R. norvegicus* and *R. rattus*), and mouse faeces and stomachs were analysed for the presence of the southern bell frog (*Litoria raniformis* (Keferstein, 1867)) (Egeter et al. 2015). The detectability of amphibians increased when using DNA methods compared to morphological methods from 0% to 50% in faeces and from 2% to 70% in stomachs (Egeter et al. 2015).

Successful species identification relies on the existing database of DNA used and whether this database covers the potential species in a sample (Collins et al. 2021; Specchia et al. 2020). Each species has a unique DNA sequence that can be databased, known as a barcode (Deagle et al. 2019). A reference library is formed with known DNA barcodes to compare and potentially match with unknown DNA fragments in a sample (Specchia et al. 2020; Thomsen & Willerslev 2015). Boyer et al. (2013) analysed the diet of the New Zealand endemic land snail, *Powelliphanta augusta* K. Walker, Trewick & G.M. Barker, 2008, from faecal samples to determine the earthworm species in the snail's diet. A DNA library from 15 earthworm clades was created (Boyer et al. 2013). The samples were

first amplified with a universal invertebrate primer followed by a second PRC protocol with modified earthworm-specific primers (Boyer et al. 2013). Thirty-five of the 46 faecal samples detected earthworms with several species detected in most faeces (Boyer et al. 2013). Overall, 13 species were detected from the library, plus three unknown distinct species, while another two species were not detected (Boyer et al. 2013). The advantage of a region-specific library is shown by Boyer et al. (2013) where many of the earthworm species were not already sequenced, therefore the outcome would have had significantly more unknown species.

Analysis of a species diet can reveal information addition to diet, such as plant-invertebrate relationship (Matheson et al. 2008), potential benefit or harm to biological control (Vink et al. 2011), the impact on commercial industries (Emami-Khoyi et al. 2016), seasonal variations dependant on migratory behaviours of other species (Schmidt et al. 2022), and competition between common and endangered species (Serite et al. 2022). Matheson et al. (2008) identified the diet of herbivorous invertebrates from a DNA reference library of 23 plant species in Israel. All reference plants were identified to be different to all other plants and successfully identified in the invertebrate diet (Matheson et al. 2008). The PCR method has a wider potential for determining plant-invertebrate relationships that influence ecosystem compositions (Matheson et al. 2008).

DNA amplified from an environmental sample will allow for the potential identification of all species present using high throughput sequencing (Boyer et al. 2013). High throughput sequencing is a method used in a variety of studies to produce multiple sequences from different species from one sample. Emami-Khoyi et al. (2016) used high throughput sequencing to identify the dietary items in the faeces of the New Zealand fur seal (*Arctocephalus forsteri* (Lesson, 1828)). A DNA library was prepared using four primer sets to amplify DNA from Chordata and Cephalopoda (Emami-Khoyi et al. 2016). The previously known diet of the New Zealand fur seal made up about 16% of the species detected by molecular analysis, revealing a larger list of diet species (Emami-Khoyi 2015). The fur seals appeared to be opportunistic feeders, taking any food that was encountered, based on the variation of the diet between colonies (Emami-Khoyi et al. 2016). Additionally, the dietary analysis of the fur seal provided information on the overlap with commercial fisheries showing only 10% of the species in the seal diet were important fisheries species (Emami-Khoyi et al. 2016).

DNA analysis of faeces has recently been used to investigate the temporal changes in the diet of the arctic fox (*Vulpes lagopus* (Linnaeus, 1758)) (Schmidt et al. 2022). By using mammal-specific primers, Schmidt et al. (2022) found that the arctic foxes' diet changed relative to the availability of prey species and did not rely on one species alone. In winter the arctic fox diet was dominated by muskox carcases (*Ovibos moschatus* (Zimmermann, 1780)) while the foxes' summer diet was dominated by

waterfowl (Schmidt et al. 2022). The molecular methods were able to identify larger prey that wouldn't be consumed whole, therefore leaving no visual evidence that would have been found with traditional visual-hand analysis (Schmidt et al. 2022).

Serite et al. (2022) compared the diet of two pipefish species, the common long-snout pipefish *Syngnathus temminckii* (Kaup, 1856) and the endangered estuarine pipefish *Syngnathus watermeyeri* (Smith, 1963) in South Africa. It was revealed that the diets did not significantly overlap, and both prefer different crustacean classes (Serite et al. 2022). *Syngnathus temminckii* primarily feed on caridean shrimp (class Malacostraca) and *S. watermeyeri* primarily feed on copepods (class Hexanauplia) (Serite et al. 2022). Serite et al. (2022) determined that the fish did not compete for food and possible competition from the common species was not causing the observed rarity of the endangered species.

The use of high throughput sequencing, along side species specific primers, has increased the knowledge of the diversity of many species' diets (Emami-Khoyi et al. 2016). High throughput sequencing and species-specific primers are faster than hand sampling (Monterroso et al. 2019), do not depend on hard parts of prey surviving digestion (Deagle et al. 2005) and have a greater output of species identified and accuracy of results (Egeter et al. 2015). Once species are sequenced and uploaded into a global database, each successive study is benefited from the previous study for each taxon. Without a central database, the DNA studies are at a disadvantage as each study would have to start with no sequences. The major disadvantage of these DNA methods are the financial cost and expertise required. However, the gain of information and decrease in labour costs often balances this out (Monterroso et al. 2019). Expertise is required with any method and when sequences are matched to species, and sharing of knowledge is ongoing between genetics and conservation biology, established analysis protocols can be followed (Monterroso et al. 2019). Increasing the knowledge of prey assists with conservation of species by promoting ecosystem management methods to support the appropriate food web (Carreon-Martinez et al. 2011), assist with habitat restoration, and provide for captive breeding programmes (Serite et al. 2022). A greater understanding of the diets of invasive species provides details on the overall ecological impact from predation and can prompt conservation action to protect a prey item that was previously unknown (Egeter et al. 2019).

1.8 Research Intent

In my thesis, I will investigate the use of a molecular approach to identifying prey items in the diet of the European hedgehog on Kaitorete Spit. Knowledge of the diet of hedgehogs will allow for an

assessment of the direct impact that hedgehogs have on the local ecosystem through predation. Predation causes a direct, detrimental impact on native fauna by driving population declines (Jones et al. 2005) and increasing competion with other insectivores (Berry 1999). Hedgehogs prey on a variety of insects, such as beetles, as well as lizard and bird species (Jones et al. 2005). Despite this, hedgehogs often pass under the scope of pest control by being deemed “cute”, and potential ecosystem harm from the hedgehog is often dismissed. Recently, wildlife control for conservation has changed to target hedgehogs more directly (Hendra 1999; Jones et al. 2021). I collaborated with existing control work by Christchurch City Council to strengthen the argument for hedgehog control. Determining the species hedgehogs’ prey on will show the species that will be protected by the removal of hedgehogs from Kaitorete Spit.

Overall, we aimed to test a method of diet analysis that has not previously been used on hedgehogs to gain a wider understanding of the diversity of the hedgehog diet. This study will also determine if these methods can be used to look for a specific species of interest. Detailed information on the diversity of species eaten and an indication of the risk this poses for the invertebrate fauna of New Zealand may change public views towards more urgent pest control management. Worldwide, the diet analysis methods in this thesis can be used, especially in the hedgehog’s natural habitat, to understand their natural diet and to assist with conservation where this species is endangered. Future studies should expand the area studied to explore dietary variation across a spatial scale and where other endangered species are at risk from hedgehog predation.

1.9 Study Site

This study analysed material collected from Kaitorete Spit, Canterbury, South Island, New Zealand (Figure 1-2) see Figure 4.1 for the specific location. Kaitorete spit runs 30 km from Tuamutu, near the mouth of the Rakaia River to the western end of Banks Peninsula (Soons et al. 1997). The spit is over 2 km at its widest point with an estimate of over 700 million m^3 of sediment (Soons et al. 1997). This narrow section of land separates Lake Ellesmere/Te Waihora from the Pacific Ocean. The material on the spit is mainly greywacke, showing its origin of river sediment from southern rivers, not eroded material from the volcanic Banks Peninsula (Speight 1930). Kaitorete "spit" is technically not a spit, but a "barrier beach" due to it being widest at the up-drift end and attached at both ends (Kirk & Lauder 2000). Despite this, the area will be referred to as 'Kaitorete Spit' as that is the most widely used name.

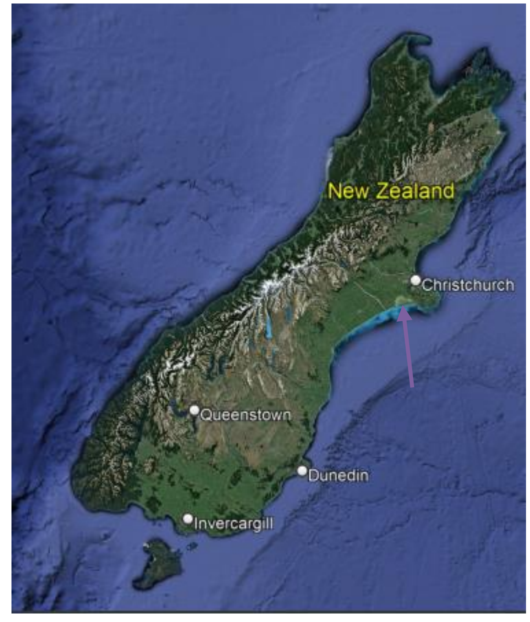


Figure 1-2. Map of the South Island of New Zealand. Yellow arrow is Kaitorete Spit. Retrieved from Google Earth.

Three main factors contributed to the formation of Kaitorete Spit. First, rapid sea level rise flooded the existing coastline that was well east of the modern line (Soons et al. 1997), which is now the continental shelf (Kirk & Lauder 2000). Second, erosion from the Rangitata River, Ashburton River and, particularly, the Rakaia River, in addition to material from erosion of the coastline, provided a range of sediment sizes from silt to sands and gravels (Kirk & Lauder 2000; Speight 1930). Third, strong northward ocean currents transported this eroded sediment to where it now sits at Kaitorete Spit (Kirk & Lauder 2000). Sediment supply is thought to have almost reached an equilibrium with the rate of erosion leaving Kaitorete Spit somewhat stable (Kirk & Lauder 2000).

The climate of Kaitorete Spit is considered the driest part of Canterbury with 400–700 mm of rain per annum (Christchurch City Council 2015). Kaitorete Spit has a unique dune ecosystem with a dominant vegetation cover of pingao (*Ficinia spiralis* (A. Rich.) Muasya et de Lange) making this site the largest population of the endemic sand binding sedge (Fake 2019). Another endangered endemic plant species at the site is shrubby tororaro (*Muehlenbeckia astonii* Petrie) with over 90% of its natural distribution on Kaitorete Spit (de Lange & Jones 2000). These species provide habitat for endangered invertebrates, including Kupe's moth (*Kupea electilis* Philpott, 1930) and the katipō

spider (*Latrodectus katipo* Powell, 1871), lizard species including McCann's skink (*Oligosoma maccanni* (Hardy, 1977)) and the Canterbury gecko (*Woodworthia brunnea* (Cope, 1869)), and ground-nesting bird species, such as the New Zealand dotterel (*Charadrius obscurus* J.F. Gmelin, 1789).

1.10 References

Berry, C. (1999). Potential interaction of hedgehogs with North Island brown kiwi at Boundary Stream Mainland Island. *Conservation Advisory Science Notes No, 268*.

Boyer, S., Wratten, S. D., Holyoake, A., Jawad, A., & Cruickshank, R. H. (2013). Using Next-Generation Sequencing to analyse the diet of a highly endangered land snail (*Powelliphanta augusta*) feeding on endemic earthworms. *PLOS ONE, 8*(9). https://doi.org/https://doi.org/10.1371/journal.pone.0075962

Brockie, R. E. (1959). Observations on the food of the hedgehog (*Erinaceus europaeus L.*) in New Zealand. *New Zealand Journal of Science, 2*(1), 121–136.

Bronnenhuber, J. E., & Wilson, C. C. (2013). Combining species-specific COI primers with environmental DNA analysis for targeted detection of rare freshwater species. *Conservation Genetics Resources, 5*(4), 971–975. https://doi.org/10.1007/s12686-013-9946-0

Bull, P. C. (1968). The Smaller Placental Mammals of Canterbury. *Natural History of Canterbury* (22), 400–402.

Campbell, P. A. (1973). The feeding behaviour of the hedgehog (*Erinaceus europaeus* L.) in pasture land in New Zealand. *New Zealand Ecological Society*(20), 35–40.

Carreon-Martinez, L., Johnson, T. B., Ludsin, S. A., & Heath, D. D. (2011). Utilization of stomach content DNA to determine diet diversity in piscivorous fishes. *Journal of Fish Biology, 78*(4), 1170–1182. https://doi.org/10.1111/j.1095-8649.2011.02925.x

Christchurch City Council. (2015). *Banks Peninsula District Plan. Chapter 6 - Reasources of the district*. https://ccc.govt.nz/the-council/plans-strategies-policies-and-bylaws/plans/christchurch-district-plan/districtplans/banks-peninsula-district-plan

Collins, K. (2015). *Hedgehogs – Cute, but lethal to native wildlife*. https://blog.forestandbird.org.nz/hedgehogs-cute-but-lethal-to-native-wildlife/

Collins, R. A., Trauzzi, G., Maltby, K. M., Gibson, T. I., Ratcliffe, F. C., Hallam, J., Rainbird, S., Maclaine, J., Henderson, P. A., Sims, D. W., Mariani, S., & Genner, M. J. (2021). Meta-Fish-Lib: A generalised, dynamic DNA reference library pipeline for metabarcoding of fishes. *Journal of Fish Biology, 99*(4), 1446–1454. https://doi.org/https://doi.org/10.1111/jfb.14852

de Lange, P., & Jones, C. (2000). Shrubby tororaro (*Muehlenbeckia astonii* Petrie) recovery plan 2000–2010. *Threatened Species Recovery Plan 31*.

Deagle, B. E., Thomas, A. C., McInnes, J. C., Clarke, L. J., Vesterinen, E. J., Clare, E. L., Kartzinel, T. R., & Eveson, J. P. (2019). Counting with DNA in metabarcoding studies: How should we convert sequence reads to dietary data? *Molecular Ecology, 28*(2), 391–406. https://doi.org/https://doi.org/10.1111/mec.14734

Deagle, B. E., Tollit, D. J., Jarman, S. N., Hindell, M. A., Trites, A. W., & Gales, N. J. (2005). Molecular scatology as a tool to study diet: analysis of prey DNA in scats from captive Steller sea lions. *Molecular Ecology, 14*(6), 1831–1842. https://doi.org/https://doi.org/10.1111/j.1365-294X.2005.02531.x

Department of Conservation. (n.d.). *Hedgehogs*. https://www.doc.govt.nz/nature/pests-and-threats/animal-pests/hedgehogs/

Di Nicola, M. R., Costa, W., & Mori, E. (2021). An evidence of asp viper (*Vipera aspis*) consumption by a western European hedgehog (*Erinaceus europaeus*) on Elba Island (Italy). *Natural history sciences.* https://doi.org/10.4081/nhs.2022.568

Dickman, C. (1987). Age-related dietary change in the European hedgehog, *Erinaceus europaeus*. *Journal of Zoology, 215*, 1–14. https://doi.org/10.1111/j.1469-7998.1988.tb04881.x

Dickman, C. R., & Huang, C. (1988). The reliability of fecal analysis as a method for determining the diet of insectivorous mammals. *Journal of Mammalogy, 69*(1), 108–113. https://doi.org/10.2307/1381753

Dowding, J. E., Elliott, M. J., & Murphy, E. C. (2015). Scats and den contents as indicators of the diet of stoats *(Mustela erminea*) in the Tasman Valley, South Canterbury, New Zealand. *New Zealand Journal of Zoology, 42*(4), 270–282. https://doi.org/10.1080/03014223.2015.1084935

Egeter, B., Bishop, P. J., & Robertson, B. C. (2015). Detecting frogs as prey in the diets of introduced mammals: a comparison between morphological and DNA-based diet analyses. *Molecular Ecology Resources, 15*(2), 306–316. https://doi.org/https://doi.org/10.1111/1755-0998.12309

Egeter, B., Roe, C., Peixoto, S., Puppo, P., Easton, L. J., Pinto, J., Bishop, P. J., & Robertson, B. C. (2019). Using molecular diet analysis to inform invasive species management: A case study of introduced rats consuming endemic New Zealand frogs. *Ecology and Evolution, 9*(9), 5032–5048. https://doi.org/https://doi.org/10.1002/ece3.4903

Emami-Khoyi, A. (2015). *Population and diet of the New Zealand fur seal (*Arctocephalus forsteri*): molecular approaches* [PhD Thesis] Lincoln University. https://hdl.handle.net/10182/6758

Emami-Khoyi, A., Hartley, D. A., Paterson, A. M., Boren, L. J., Cruickshank, R. H., Ross, J. G., Murphy, E. C., & Else, T.-A. (2016). Identifying prey items from New Zealand fur seal (*Arctocephalus forsteri*) faeces using massive parallel sequencing. *Conservation Genetics Resources, 8*(3), 343–352. https://doi.org/10.1007/s12686-016-0560-9

Fake, M. (2019). *Unmanned aerial system derived multi-spectral imagery for the monitoring of coastal dune plant communities* [Masters, Lincoln University]. https://researcharchive.lincoln.ac.nz/handle/10182/10635

Foster, N. J., Maloney, R. F., Recio, M. R., Seddon, P. J., & van Heezik, Y. (2021). European hedgehogs rear young and enter hibernation in New Zealand's alpine zones. *New Zealand Journal of Ecology, 45*(2), 1–6. https://doi.org/https://doi.org/10.20417/nzjecol.45.52

Fountain, E., Pugh, A., & Bowie, M. H. (2013). *What do predators eat for supper? Burkes Pass Scenic Reserve predator stomach content analysis for 2010–2011*. https://researcharchive.lincoln.ac.nz/handle/10182/6814

Gazzard, A., & Baker, P. J. (2020). Patterns of feeding by householders affect activity of hedgehogs (*Erinaceus europaeus*) during the hibernation period. *Animals, 10*(8), 1344. https://www.mdpi.com/2076-2615/10/8/1344

Gazzard, A., Yarnell, R. W., & Baker, P. J. (2022). Fine-scale habitat selection of a small mammalian urban adapter: the West European hedgehog (*Erinaceus europaeus*). *Mammalian Biology, 102*(2), 387–403. https://doi.org/10.1007/s42991-022-00251-5

Gonçalves, J., Marks, C. A., Obendorf, D., Amorim, A., & Pereira, F. (2014). The risks of using “species-specific” PCR assays in wildlife research: The case of red fox (*Vulpes vulpes*) identification in Tasmania. *Forensic Science International: Genetics, 11*, 9–11. https://doi.org/https://doi.org/10.1016/j.fsigen.2014.03.009

Hendra, R. (1999). *Seasonal abundance patterns and dietary preferences of hedgehogs at Trounson Kauri Park.* Conservation Advisory Science Notes no. 267 https://www.doc.govt.nz/documents/science-and-technical/casn267.pdf

Hof, A. R., & Bright, P. W. (2010). The value of agri-environment schemes for macro-invertebrate feeders: hedgehogs on arable farms in Britain. *Animal Conservation, 13*(5), 467–473. https://doi.org/https://doi.org/10.1111/j.1469-1795.2010.00359.x

Jones, C., Garvey, P., Graham, B., & Ross, J. (2021). *Hedgehog (*Erinaceus europaeus*) control tools: Relative attractiveness of potential lures and effects of aperture size on device access.* Department of Pest Management and Conservation, Contract Report: LC3985. https://researcharchive.lincoln.ac.nz/handle/10182/13914

Jones, C., Moss, K., & Sanders, M. (2005). Diet of hedgehogs (*Erinaceus europaeus*) in the upper Waitaki Basin, New Zealand: Implications for conservation. *New Zealand Journal of Ecology, 29*(1), 29–35. https://newzealandecology.org/nzje/2244

Jones, C., & Norbury, G. (2011). Feeding selectivity of introduced hedgehogs (*Erinaceus europaeus*) in a dryland habitat, South Island, New Zealand. *Acta Theriologica, 56*(1), 45–51. https://doi.org/10.1007/s13364-010-0009-6

King, C. (2023). Abundance and dynamics of small mammals in New Zealand: sequential invasions into an island ecosystem like no other. *Life (Basel), 13*(1). https://doi.org/10.3390/life13010156

King, C. M., Innes, J. G., Flux, M., Kimberley, M. O., Leathwick, J. R., & Williams, D. S. (1996). Distribution and abundance of small mammals in relation to habitat in Pureora Forest Park. *New Zealand Journal of Ecology, 20*(2), 215–240.

King, C. M., & Morris, R. (2008). *A photographic guide to mammals of New Zealand*. New Holland.

Kirk, R. M., & Lauder, G. A. (2000). *Significant coastal lagoon systems in the South Island, New Zealand*. https://www.doc.govt.nz/globalassets/documents/science-and-technical/sfc146.pdf

Marco-Tresserras, J., & López-Iborra, G. M. (2023). Nesting ecology of european hedgehogs (*erinaceus europaeus*) in urban areas in southeast spain: nest habitat use and characteristics. *Animals, 13*(15), 2453. https://doi.org/https://doi.org/10.3390/ani13152453

Marris, J. W. M. (2000). The beetle (Coleoptera) fauna of the Antipodes Islands, with comments on the impact of mice and an annotated checklist of the insect and arachnid fauna. *Journal of the Royal Society of New Zealand, 30*(2), 169–195. https://doi.org/10.1080/03014223.2000.9517616

Matheson, C., Muller, G., Junnila, A., Vernon, K., Miller, M., Greenblatt, C., Hausmann, A., & Schlein, Y. (2008). A PCR method for detection of plant meals from the gut of insects. *Organisms Diversity & Evolution, 7*, 294–303. https://doi.org/10.1016/j.ode.2006.09.002

Micol, T., Doncaster, C. P., & Mackinlay, L. A. (1994). Correlates of local variation in the abundance of hedgehogs (*Erinaceus europaeus)*. *Journal of Animal Ecology, 63*(4), 851–860. https://doi.org/10.2307/5262

Montague, T. L. (2000). *The brushtail possum : biology, impact and management of an introduced marsupial*. Lincoln, N.Z. Manaaki Whenua Press.

Monterroso, P., Godinho, R., Oliveira, T., Ferreras, P., Kelly, M. J., Morin, D. J., Waits, L. P., Alves, P. C., & Mills, L. S. (2019). Feeding ecological knowledge: the underutilised power of faecal DNA approaches for carnivore diet analysis. *Mammal Review, 49*(2), 97–112. https://doi.org/https://doi.org/10.1111/mam.12144

Morris, P. (1985). The effects of supplementary feeding on movement of hedgehogs (*Erinaceus europaeus*). *Mammal Review, 15*(1), 23–33. https://doi.org/10.1111/j.1365-2907.1985.tb00383.x

National Pest Control Agencies. (2018). *A8 Pest Mustelids, Monitoring and Control* https://www.bionet.nz/assets/Uploads/A8-Pest-Mustelids-2018-04-LR.pdf

Nottingham, C. M., Glen, A. S., & Stanley, M. C. (2019). Snacks in the city: The diet of hedgehogs in Auckland urban forest fragments. *New Zealand Journal of Ecology, 43*(2). https://doi.org/10.20417/nzjecol.43.24

Pipek, P. (2020). Independent introductions of hedgehogs to the North and South Island of New Zealand. *New Zealand Journal of Ecology, 44*(1), 30p.

Pracy, L. T. (1974). *Introduction and liberation of the opossum (*Trichosurus vulpecula*) into New Zealand* (2nd ed.). New Zealand Forest Service.

Predator Free NZ. (n.d.). *DOC 150/200/250 traps*. https://predatorfreenz.org/toolkits/trapping-baiting-toolkit/trap-bait-and-equipment-tips/how-to-choose-the-right-trap/doc-150-200-250-traps/

Rautio, A., Isomursu, M., Valtonen, A., Hirvelä-Koski, V., & Kunnasranta, M. (2016). Mortality, diseases and diet of European hedgehogs (*Erinaceus europaeus*) in an urban environment in Finland. *Mammal Research, 61*(2), 161–169. https://doi.org/10.1007/s13364-015-0256-7

Sanders, M. D., & Maloney, R. F. (2002). Causes of mortality at nests of ground-nesting birds in the Upper Waitaki Basin, South Island, New Zealand: a 5-year video study. *Biological Conservation, 106*(2), 225–236. https://doi.org/https://doi.org/10.1016/S0006-3207(01)00248-8

Schlesselmann, A.-K. V., O'Donnell, C. F. J., Monks, J. M., & Robertson, B. C. (2018). Clearing islands as refugia for black-fronted tern (*Chlidonias albostriatus*) breeding colonies in braided rivers. *New Zealand Journal of Ecology, 42*(2), 137–148. https://www.jstor.org/stable/26538105

Schmidt, N. M., Roslin, T., Hansen, L. H., Gilg, O., Lang, J., Sittler, B., Hansen, J., Bollache, L., & Vesterinen, E. (2022). Spatio-temporal patterns in arctic fox (*Vulpes alopex*) diets revealed by molecular analysis of scats from Northeast Greenland. *Polar Science, 32*, 100838. https://doi.org/https://doi.org/10.1016/j.polar.2022.100838

Serite, C. P., Emami-Khoyi, A., Ntshudisane, O. K., James, N. C., van Vuuren, B. J., Bodill, T., Cowley, P. D., Whitfield, A. K., & Teske, P. R. (2022). eDNA metabarcoding vs metagenomics: an assessment of dietary competition in two estuarine pipefishes. *bioRxiv*, 2021.2001.2005.425398. https://doi.org/10.1101/2021.01.05.425398

Soons, J. M., Shulmeister, J., & Holt, S. (1997). The Holocene evolution of a well nourished gravelly barrier and lagoon complex, Kaitorete "Spit", Canterbury, New Zealand. *Marine Geology, 138*(1), 69–90. https://doi.org/https://doi.org/10.1016/S0025-3227(97)00003-0

South, K. E., Haynes, K., & Jackson, A. C. (2020). Hibernation patterns of the european hedgehog, *Erinaceus europaeus,* at a cornish rescue centre. *Animals, 10*(8), 1418. https://www.mdpi.com/2076-2615/10/8/1418

Specchia, V., Tzafesta, E., Marini, G., Scarcella, S., Simona, D. A., & Pinna, M. (2020). Gap analysis for DNA Barcode Reference Libraries for aquatic macroinvertebrate species in the Apulia Region (Southeast of Italy). *Journal of Marine Science and Engineering, 8*(7), 538. https://doi.org/https://doi.org/10.3390/jmse8070538

Speight, R. (1930). The Lake Ellesmere Spit with map, sections and photographs. *Transactions and Proceedings of the Royal Society of New Zealand, 61*, 147–177. https://paperspast.natlib.govt.nz/periodicals/TPRSNZ1930-61.2.5.1.7

Taylor, R. H. (1975). What limits kiore (*Rattus exulans*) distribution in New Zealand? *New Zealand Journal of Zoology, 2*(4), 473–477. https://doi.org/10.1080/03014223.1975.9517888

Thomsen, P. F., & Willerslev, E. (2015). Environmental DNA – An emerging tool in conservation for monitoring past and present biodiversity. *Biological Conservation, 183*, 4–18. https://doi.org/https://doi.org/10.1016/j.biocon.2014.11.019

Vink, C., McNeill, M., Winder, L., Kean, J., & Phillips, C. (2011). PCR analyses of gut contents of pasture arthropods. In *Paddock to PCR - Demystifying molecular biology for practical plant protection* (pp. 125–134). New Zealand Plant Protection Society.

Vink, C. J., & Kean, J. M. (2013). PCR gut analysis reveals that *Tenuiphantes tenuis* (Araneae: Linyphiidae) is a potentially significant predator of Argentine stem weevil, *Listronotus bonariensis* (Coleoptera: Curculionidae), in New Zealand pastures. *New Zealand Journal of Zoology, 40*(4), 304–313. https://doi.org/10.1080/03014223.2013.794847

Yalden, D. W. (1976). The food of the hedgehog in England. *Acta Theriologica, 21*(30), 401–424 https://doi.org/10.4098/at.arch.76-39

Young, L. M., Marris, J. W. M., & Pawson, S. M. (2008). Back from extinction: rediscovery of the Canterbury knobbled weevil *Hadramphus tuberculatus* (Pascoe 1877) (Coleoptera: Curculionidae), with a review of its historical distribution. *New Zealand Journal of Ecology, 35*, 323–330. https://doi.org/10.1080/03014220809510129

Young, R., Davison, J., Trewby, I., Wilson, G., Delahay, R., & Doncaster, C. (2006). Abundance of hedgehogs (*Erinaceus europaeus*) in relation to the density and abundance of badgers (*Meles meles*). *Journal of Zoology, 269*, 349–356. https://doi.org/10.1111/j.1469-7998.2006.00078.x

Chapter 2

Developing a DNA method to detect a known dietary item (mealworms, *Tenebrio molitor* larvae) in hedgehog (*Erinaceus europaeus*) faeces.

2.1 Introduction

Ecological studies often aim to investigate the impact caused by a single predatory species, or the effect of the environment on a specific prey species. To determine these interactions, monitoring techniques are developed to detect the prey species. Monitoring is important to observe prey species' presence and abundance over time, especially with disturbances, such as habitat clearance (Schneyer 2002), pest incursion (Duncan et al. 2011) and climate change (Freeman et al. 2018). Invertebrates are commonly monitored by hand collecting, leaf litter sampling, and encounter traps, such as pitfall and light traps (Marris 2000). Unfortunately, these methods do not provide any information about predation by introduced mammals. Diet analysis is commonly done through morphological identification of fragments remaining in the stomach (Brockie 1958; Fountain et al. 2013) and faeces (Dickman & Huang 1988) of animals. Recent studies have begun using DNA as a tool to detect species in stomach (Vink et al. 2011) and faecal (Emami-Khoyi et al. 2016) samples. DNA methods have the advantage of detecting soft body parts that are often missed by morphological analysis (Egeter 2014; Gosselin et al. 2017).

To target a known species, DNA can be analysed from environmental samples (eDNA), such as soil, sediment, and water (Thomsen & Willerslev 2015). Environmental DNA from waterways can return the DNA of the species associated with the water catchment at the sample site and upstream (Lear et al. 2017). Environmental DNA has been used to detect rare freshwater species in the North American Laurentian Great Lakes ecosystems, where eDNA is used to monitor the presence of endangered and invasive species (Bronnenhuber & Wilson 2013). Animal faeces are another source of eDNA that contains species present in the diet of predators and herbivores that are specialist or generalist feeders (Emami-Khoyi et al. 2016). Species in the diet can be detected through the polymerase chain reaction (PCR) that amplifies target DNA. PCR uses short sections of DNA, known as primer pairs, designed to match target genes from a group of closely related organisms or a single species.

Species-specific PCR primers can be used to detect species of interest in faecal samples from a range of taxa, including amphibians (Egeter et al. 2015), fish (Bronnenhuber & Wilson 2013; Deagle et al. 2005), invertebrates (Vink & Kean 2013) and mammals (Gonçalves et al. 2014). Dietary analysis in

combination with prey population models is often used to predict the impact of an invasive species, which may be herbivory, predation, or competition (Park 2004). Predation has a direct impact on the population of species and if unknown, no preventative measures can be implemented to protect the native species.

The European hedgehog (*Erinaceus europaeus*) is an introduced invasive species in New Zealand and is a generalist omnivore, although it is thought to primarily feed on invertebrates (Dierenfeld 2009; Jones et al. 2005). Environmental DNA is a useful tool for detecting species in a hedgehog's diet because they have a quick gut-passage time of 6–9 hrs (Egeter 2014), therefore the faecal sample shows recent evidence of feeding behaviour. Diet analysis based on morphology has already shown a large diversity in hedgehog faeces (Egeter 2014) and stomachs (Fountain et al. 2013) but other studies have shown that just morphology can miss some species that are hard to detect due to being soft-bodied (Egeter et al. 2015; Gosselin et al. 2017).

Hedgehog faecal content has been analysed to detect predation on southern bell frogs (*Litoria raniformis*) during a feeding experiment that used specific PCR primers (Egeter et al. 2015). The detectability of southern bell frogs using morphological analysis and DNA-based analysis increased from 0% to 78% for faeces and 8% to 75% for stomachs respectively (Egeter et al. 2015). The design of species-specific primers aims to prevent the detection of non-target species (false positive) in the presence of other DNA in samples, which is important to ensure true results. A review of a primer pair supposedly specific for red fox (*Vilpes vulpes*) by Gonçalves et al. (2014) showed that insufficient non-target testing had been done. The DNA of other species such as European rabbit (*Oryctolagus cuniculus*) European hare (*Lepus europaeus*), Iberian hare (*Lepus granatensis*), cattle (*Bos taurus*) and pig (*Sus scrofa*) were amplified using the primer pair. This is a concern because wrong detection may prompt expensive eradication programs if a false positive indicates an invasive species is present, while the target species may not be (Gonçalves et al. 2014). The primers must be designed for a region of DNA that is unique to the target species and will not select non-target DNA during PCR and show that specific species can be detected in a hedgehog's diet.

This chapter focuses on the development of DNA methods to detect a single species (mealworms (*Tenebrio molitor* Linnaeus, 1758) in the diet of captive hedgehogs. This will include methods for the captive feeding trail, designing the species-specific DNA primers to target mealworms and designing the PCR profile for amplifying mealworm DNA from hedgehog faecal content. The PCR profile was based on a study of the diet of the New Zealand fur seal (Emami-Khoyi et al. 2016).

2.2 Objectives

Construct a DNA protocol to detect a known dietary item in hedgehog faecal content.

2.2.1 Undertake a trial feeding mealworms to hedgehogs in a controlled environment.

2.2.2 Design primers to detect mealworms from raw mealworm DNA.

2.2.3 Determine PCR parameters to detect mealworms in hedgehog feaces.

2.3 Methods

2.3.1 Mealworm Feeding Trial

The mealworm feeding trial was undertaken at the mammal trial pens at the Johnstone Memorial Laboratory (JML), Lincoln University, in collaboration with Manaaki Whenua Landcare Research. These pens have an area of about 3 m^2 with a sheltered corner and grass making up the floor (Figure 2-1). All hedgehogs were kept in separate pens in the same area and were, therefore, exposed to the same climatic conditions. Eleven hedgehogs were caught from the same area in the wild, following Landcare Research regulations, and housed for a minimum of four days before the experiment to acclimatise. Their diet during this period was the standard maintenance diet used by JML consisting of an abundant supply of dry Whiskas® cat food (Whiskas® Adult Dry Cat Food Meaty Selections) with a corn base and real chicken meat in the biscuits. Before the trial each hedgehog was ear tagged, weighed, sexed, and had a pre-trial health check. The pens were cleared of all existing faeces before the trial. Each trial ran over four nights of feeding and on each following day, fresh faeces were collected. The hedgehogs were fed one of three diets for the whole trial period. Three hedgehogs were fed the normal maintenance diet, four were fed one live mealworm in addition to the normal maintenance diet, and four were fed 10 live mealworms in addition to the normal maintenance diet. The hedgehogs were fed in a standard double-sided cat dish (Figure 2-2), with one side for 50 g of cat food, and the other for mealworms, to allow easy monitoring of eaten mealworms. Each day any remaining mealworms were removed before being replaced with fresh mealworms and the cat food was topped up. During the trial, the pens were searched daily for faeces and collected into separate pottles with labels of date and hedgehog ID number. The collected faecal content was immediately frozen until DNA analysis.

Ethics

Animal ethics were approved internally by Manaaki Whenua Landcare Research (AEC Code: 23/01/01; Job Number: PRJ3234) and provided for the Lincoln University Animal Ethics Committee.

Figure 2-1. Pen used to hold hedgehogs during the mealworm feeding trial in February 2023.

Figure 2-2. Feeding dishes for the mealworm feeding trial with 10, one or no mealworms and the maintenance diet.

2.3.2 Primer Design

A primer pair was designed specifically for mealworms *(Tenebrio molitor)* from existing sequences of the cytochrome c oxidase subunit I (COI) mitochondrial gene from GenBank using the Primer Blast tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). The primers (Mealworm F1 5'-TCCACCCTTATCCTCTAATATCGC-3' and Mealworm R1 5'-ACTGCTCACACGAATAACGG-3') amplified a 177-bp COI sequence segment (Table 2-2).

The primers were tested against DNA sourced from individual mealworms and non-target species that could be present during the feeding trial. The non-target DNA included Coleoptera (*Mimopeus* sp. Pascoe, 1866, *Carpophilus hemipterus* (Linnaeus, 1758), Hydrophilidae Latreille, 1802, *Laemostenus complanatus* (Dejean, 1828)), Diptera (*Musca domestica* De Geer, 1776, *Sylvicola* sp. Harris, 1780), Dermaptera (*Nesogaster halli* Hincks, 1949), Araneae (*Steatoda capensis* Hann, 1990), European hedgehog (*Erinaceus europaeus*), as well as DNA found in the Whiskas® Cat food. These

non-target samples were chosen based on potential exposure during the feeding trial (hedgehog and cat food), being closely related to the target DNA (*Mimopeus* sp.) or extracted DNA that was available in the Pest-management and Conservation Department's molecular ecology laboratory at Lincoln University to decrease extraction costs.

Sample DNA of mealworms and the non-targets listed above was extracted using a QIAGEN DNeasy Blood & Tissue Kit. Insect specimens were either freshly killed, frozen, or stored in 70% ethanol until extraction from one or two legs, hedgehog DNA was taken from an ear clipping of a frozen hedgehog, and the Whiskas® cat food was extracted from one biscuit of each type from a fresh bag of food. PCR amplification was done using an Applied Biosystems Veriti 96 Well Thermal Cycler with 1 µL of template DNA, 10 µL of thermo scientific Dream Taq Green PCR Master Mix (2x), 1 µL of each of the forward and reverse primers (10 µM concentration), and 7 µL of H_2O to form a 20 µL solution. The cycling profile included initial denaturation at 94 °C for three minutes, then 35 cycles of 94 °C denaturing for 30 seconds, annealing for 30 seconds at 50 °C, 55 °C or 60 °C and extension at 72 °C for one minute followed by a final extension at 72 °C for five minutes. PCR products were run on a 1% Agarose gel dyed with INtRON RedSafe™ nucleic acid staining solution for 30 minutes at 90 volts to visualise the amplified DNA. The highest working annealing temperature was 60 °C. A higher temperature minimised the chance of primers annealing to non-target DNA. This temperature was used for the initial analysis of non-target species.

For non-target DNA, the PCR and gel protocol was also the same as above with the initial annealing temperature of 60 °C. Any non-target species that were detected were re-analysed at increased temperatures of 60 °C, 62 °C, and 64 °C to determine when they would not be amplified. The temperature that only detected mealworm DNA was 64 °C and this was used for analysis of the faeces from the feeding trial.

2.3.3 DNA Analysis of faecal content

DNA from the faecal samples were extracted using a QIAGEN DNeasy PowerMax soil kit recommended by previous research on seal diets (Emami-Khoyi 2015). Modifications were made to the Power Max soil kit protocol at step 4 where instead of vortexing for 10 minutes on high, the samples were placed into a 1600 Mini G SPEX Sample Prep for 10 minutes at 1500 RPM. The faeces used for analysis were from hedgehogs that had eaten at least one mealworm the night prior. When the faeces from a night weighed more than 10 g, all the faeces from that night were mixed with a sterile pipette and a 10 g sub-sample was taken. PCR amplification was done according to the procedure above with an annealing temperature of 64 °C, determined by the primer design testing.

PCR products were then run on a 50 mL 1% Agarose gel for 30 minutes at 90 volts to visualise the amplified DNA.

2.3.4 Sequencing Mealworm DNA

The post-PCR product was prepared for Sanger sequencing following the ExoSAP-IT PCR product clean-up protocol. For sequencing, 1 µL of cleaned product and 0.5 µL of primer was used in the Lincoln University Sequencing facility. Both the forward and reversed directions were sequenced with the appropriate primer designed above.

2.4 Results

2.4.1 Mealworm Trial Feeding Rates

During the four feeding nights, four of the 11 hedgehogs successfully fed on mealworms (Table 2-1) with a total of 9 nights feeding.

Table 2-1. Mealworm feeding trial results. *Hedgehog 11 broke into Hedgehog 9's pen.

Hedgehog	Mealworms	Night 1	Night 2	Night 3	Night 4
1	0				
6	0				
7	0				
3	1				
4	1				
5	1	1	1	1	1
11	1				
2	10		5	2	
8	10		5	3	
9	10		10*	0	
15	10				

2.4.2 Primer Design

Table 2-2. Criteria for the specific primer design of the mealworm COI gene (*Tenebrio molitor*).

Primer Type	Primer Sequence	Base Length (bp)	%GC	Tm (°C)	Number of Amplicons
Forward Primer	5'-TCCACCCTTATCCTCTAATATCGC-3'	24	46	59	177
Reverse Primer	5'-ACTGCTCACACGAATAACGG-3'	20	50	58	

The primers, with the parameters shown in Table 22, were tested at a range of temperatures and successfully amplified mealworms DNA at 50 °C, 55 °C and 60 °C.

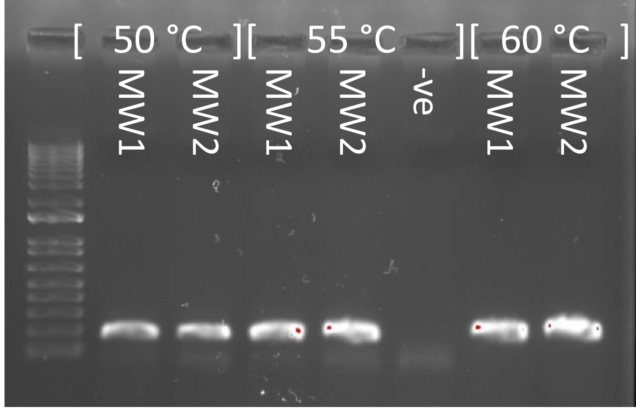


Figure 2-3. Mealworm-specific primer testing on mealworm (MW) DNA at 50 °C, 55 °C, and 60 °C.

2.4.3 Non-Target Primer Testing

The primers were tested against non-target species (Table 2-3Table 4-2). Two non-target species were amplified, E64 (Coleoptera) and E67 (Diptera). These samples were then tested at higher PCR annealing temperatures and no non-targets were detected at 64 °C.

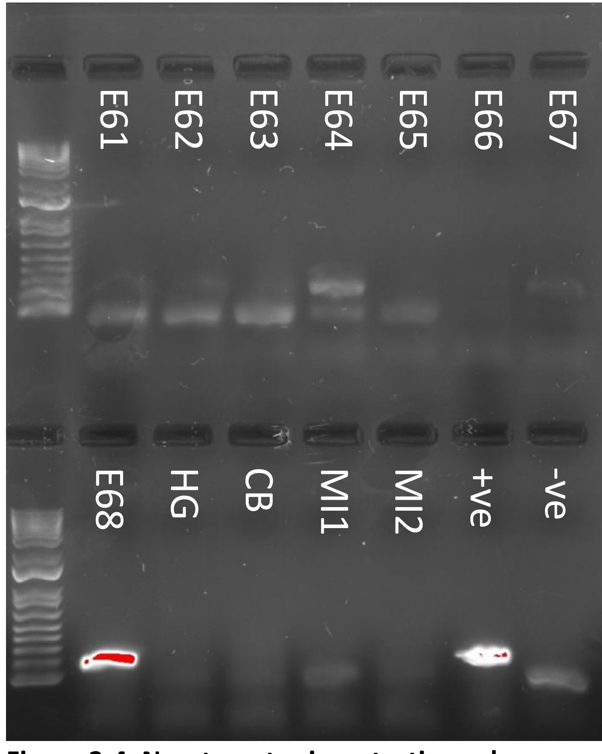


Figure 2-4. Non-target primer testing gel electrophoresis at an annealing temperature of 60 °C

Table 2-3. Lab code and species name of non-target species against the mealworm-specific primer.

Lab code	Species/Item	Order
E61	*Carpophilus hemipterus*	Coleoptera
E62	*Nesogaster halli*	Dermaptera
E63	Hydrophilidae	Coleoptera
E64	*Laemostenus complanatus*	Coleoptera
E65	*Musca domestica*	Diptera
E66	*Steatoda capensis*	Araneae
E67	*Sylvicola* sp.	Diptera
E68	*Tenebrio molitor*	Coleoptera
HG	*Erinaceus europaeus* (Hedgehog)	Eulipotyphla
CB	Whiskas® Cat Food	
MI1	*Mimopeus* species 1	Coleoptera
MI2	*Mimopeus* species 2	Coleoptera
+ve	Positive Control MW1 (Figure 2-3)	Coleoptera
-ve	Negative Control	H_2O

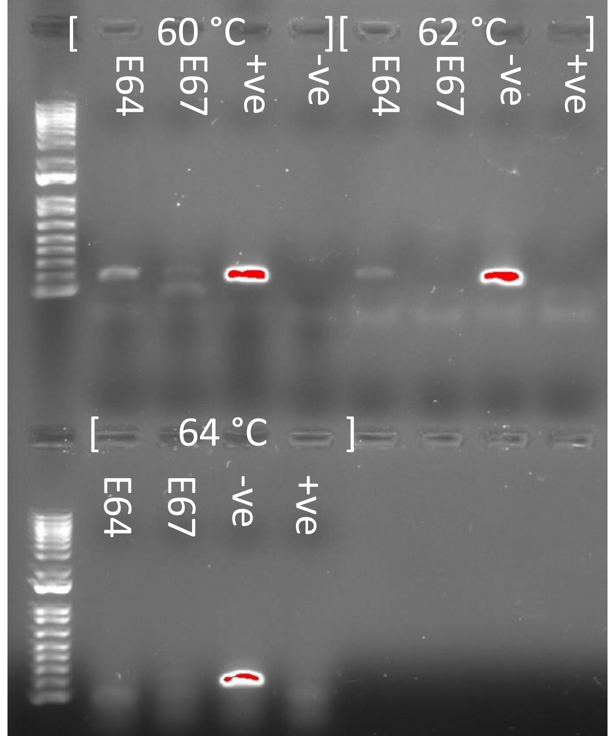


Figure 2-5. Non-Target primer testing gel electrophoresis at an annealing temperature of 60 °C, 62 °C, and 64 °C of the false positives.

2.4.4 Faecal analysis

Mealworm DNA was successfully amplified from a single faecal sample (Figure 2-6) that weighed 10.2 g and no subsample was taken. All other samples either weighed <6.5 g or a 10 g sub-sample was taken (Table 2-4). The positive fragment was confirmed with sequencing, see 2.4.5.

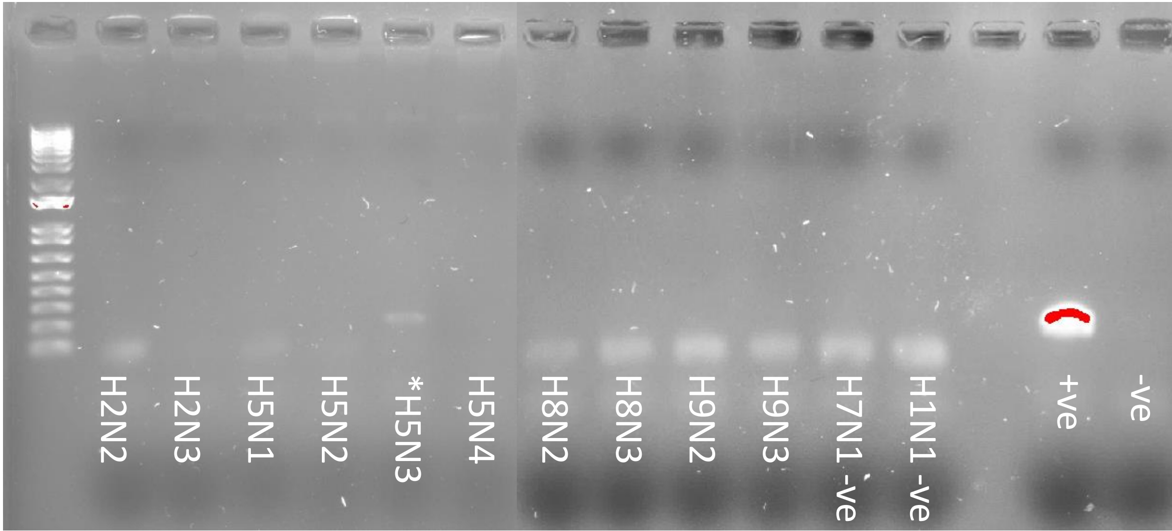


Figure 2-6. Gel electrophoresis output of PCR of faeces from hedgehogs fed mealworms. H = Hedgehog, N = Night of feeding. *H5N3 was the only faecal sample with a positive detection.

Table 2-4. Diet design, weight, and proportion of faecal subsamples from the mealworm feeding trial.

Faecal Code	Treatment	Eaten	Total Weight (g)	Subsample	Proportion
H2N2	10	5	1.37	1.37	100%
H2N3	10	2	16.57	9.32	56%
H5N1	1	1	18.62	9.02	48%
H5N2	1	1	6.48	6.48	100%
*H5N3	1	1	10.2	10.2	100%
H5N4	1	1	3.47	3.47	100%
H8N2	10	5	27.2	9.5	35%
H8N3	10	3	11.26	9.3	83%
H9N2	10	10	20.03	10.6	53%
H9N3	10	0	4.86	4.86	100%
H1N1 -ve	0	0	9.9	9.9	100%
H7N1 -ve	0	0	2.2	2.2	100%

Sequence PCR output

The sequence of sample H5N3 returned at 100% *Tenebrio molitor* for 32 published sequences when blasted using the GenBank database, including the complete *T. molitor* mitochondrion genome. The sequence is
"CCATGGAGGAGCATCTGTCGATTTAGCAATTTTCAGGCTACATCTAGCAGGGATTTCGTCAATCCTG GGGGCCGTAAATTTTATTACAACAGTAATCAACATACGACCACAGGGCATAACGTTCGATCGAATA".

2.5 Discussion

This chapter shows that mealworm DNA can be amplified from hedgehog faeces using a species-specific primer pair. The confirmation of mealworm DNA through Sanger sequencing (2.4.5), in addition to electrophoresis gel visualisation (2.4.4), unequivocally confirms that this method works under the conditions of the study. The success of the methods confirm that detectable fragments of DNA can be found in hedgehog faeces and that DNA is not completely denatured in the stomach. Mealworms were chosen for this trial because wild hedgehog diets show a high proportion of beetle consumption (Jones et al. 2005; Jones & Norbury 2011). In captivity, mealworms are an easily accessible invertebrate that is often fed to hedgehogs and other general insectivores in small amounts (Allen 1989). Mealworms were a small part of the diet during the sampling and the trial was over a short period of four days, therefore, avoiding any health issues from long-term feeding (Allen 1989).

The non-target testing indicated a false positive of the primer pair, amplifying another coleopteran species (*Laemostenus complanatus*) at a lower annealing temperature of 60 °C (2.4.3. Figure 2-4). Mealworms and *L. complanatus* are not closely related, being from the families Tenebrionidae and Carabidae respectively (McKenna et al. 2019), indicating that the target section of the COI gene is similar between these species. Increasing the annealing temperature to 64 °C prevented amplification of *L. complanatus* while still detecting mealworms (2.4.3. Figure 2-5), concluding that a higher annealing temperature is required during PCR than suggested by the primer melting temperature (Tm) (58–59 °C). The annealing temperature is recommended to be 5–10 °C lower than the primer Tm because of the risk of low primer annealing rates and decreasing the PCR yield at a higher temperature (Tabarzad et al. 2014; Wu et al. 1991). To avoid non-target detections the temperature recommendation was not followed and proved to be suitable to detect mealworm DNA.

A positive detection of mealworm DNA in hedgehog faeces is a promising proof of concept, but to enhance the methods, further trials could be done. Such trials were beyond the time scope for this

study as hedgehogs began to hibernate and feeding rates decreased. Our results showed mealworm DNA in one of the 10 hedgehog faeces analysed. This could be due to poor PCR design, low volume of target DNA in the diet, or slow gut-passage time of the hedgehogs. Many modifications can be made to enhance the PCR protocol including redesigning the primers, changing the annealing temperature, and changing the number of PCR cycles. Redesigning the primers for a different section of COI, or a different gene may prevent the amplification of non-target species. Primers must target a region that differs significantly between species, making it hard to design reliable primers (Gonçalves et al. 2014). This study also had 35 cycles for amplification, while other studies have found that with 25 or more cycles, non-target DNA was detected in gels due to opportunistic mispairing (Citartan et al. 2012; Tabarzad et al. 2014). As this was a proof of concept, when no non-targets were detected with a higher annealing temperature and the target DNA was detected, we did not need to redesign the primers or PCR protocol. With another primer pair, the PCR protocol used in this study may not work, therefore these other options should be considered.

The volume of target DNA in the diet of the hedgehogs in the feeding trial was only 1–10 larvae (10 larvae = ~1.5 g) alongside 50 g of cat food as a maintenance diet. It is possible that the hedgehogs were over-fed with their maintenance diet and were not motivated to explore new food. This lack of motivation is suggested by hedgehogs eating the available mealworms in only 10 of 36 potential feeding times. To increase the feeding events, the trial could be extended to expose the hedgehogs to the new food for longer. Campbell (1975) studied the feeding behaviour of captive hedgehogs and allowed nine weeks for animals to condition to being in captivity. After this period the feeding trial showed that captive hedgehogs had similar feeding rhythms to wild hedgehogs with the highest activity between 9–11 pm (Campbell 1973). Alongside a longer trial, the volume of a maintenance diet could be decreased to promote more explorative feeding by the hedgehogs. Not all mealworms were eaten, therefore increasing the number at the start of the feeding trial, will likely not increase the number eaten. Small volumes of prey species have been shown to be as reliably detected as abundant dietary items when using species-specific primers (Deagle et al. 2005). The limitation of the feeding trial is likely due to the time exposed to a new food, rather than the amount in the diet. Other common insects used as pet food should be tested in further trials to determine if another species may be more suitable for feeding trial success, e.g., hedgehogs may feed more readily on adult insects. It is possible that the cat food used for the maintenance diet contained other insect DNA; however, this should not impede the results of my study where only mealworms were being sought after and were not detected from the non-target testing. The ingredients of the maintenance diet should be further tested for any future studies looking for other dietary items.

Hedgehog gut-passage time is 6–9 hours, with some extremes of 12 hours (Egeter 2014). It is possible that hedgehogs fed on mealworms and did not expel their colon contents until the following evening. Campbell (1975) observed captive hedgehogs normally first groomed and then defecated after emerging from their nests. To account for delayed gut-passage time and first emergence defecation in future studies, the faeces from the next feeding night of successful feeding could also be analysed even if no mealworms were eaten that night. Similar methods were used by Egeter (2014) who collected hedgehog faeces up to 30 hours after ingestion of the feeding trial diet (frogs *Litoria raniformis*). My project did not have the budget to analyse more than 12 hedgehog faeces so only faeces produced the day of mealworms being eaten were analysed.

Another limitation of this project that stemmed from the limiting samples to 12, was the effect of sub-sampling. The QIAGEN DNeasy PowerMax soil kit only processes 10 g of a sample in each extraction. Five of the hedgehog faeces weighted ≤ 10g, while the remining five samples weighed >10 g, therefore a subsample of 10 g was used. The issue with sub-sampling is the risk of not homogenising (combining) the sample completely, leading to a non-representative sample or different outcomes from the same sample (Rosel & Kocher 2002). Sampling all contents from faeces would be ideal to overcome biases created by subsampling or ensure to completely homogenise samples mechanically not manually.

Once the presence of extractable DNA in the hedgehog diet is resolved, the next step is to examine the content of wild hedgehog faeces. Such an approach will determine if known endemic species are being preyed on by creating other species-specific primers. The primer methodology in this chapter was a proof-of-concept trial to determine a low-cost method to confirm that insect DNA could be detected in hedgehog faecal content. More expensive methods, such as high throughput sequencing (HTS), can processes all the sequences in a sample that represent multiple species. Chapter 5 uses HTS to investigate wild hedgehog diets from faecal and colon samples.

2.6 Conclusion

The result of this study shows that mealworm DNA can be detected in hedgehog faeces using species-specific primers. To avoid non-target amplification during PCR the annealing temperature was increased above the recommended primer melting temperature; however, this increase in temperature was still suitable to detect mealworm DNA. Future studies should look at trial length, next-day faeces and alternative target species to ensure a robust methodology has been developed, before being tested on wild hedgehog faeces. This methodology has the potential to monitor the predation impact of hedgehogs on a specific species of conservation interest that is endangered or geographically restricted.

References

Allen, M. E. (1989). *Nutritional asppects of insectivory* [PhD Dissertation] Michigan State University. https://doi.org/doi:10.25335/M5SB3X582

Brockie, B. (1958). *The ecology of the hedgehog (*Erinaceus europaeus *L.) in Wellington, New Zealand : [a thesis] submitted for the degree of Master of Science in Zoology at the Victoria University of Wellington* https://mro.massey.ac.nz/server/api/core/bitstreams/594fd3e7-cc8f-41e8-86cb-6f2b49bad97b/content

Bronnenhuber, J. E., & Wilson, C. C. (2013). Combining species-specific COI primers with environmental DNA analysis for targeted detection of rare freshwater species. *Conservation Genetics Resources, 5*(4), 971–975. https://doi.org/10.1007/s12686-013-9946-0

Campbell, P. A. (1973). *Feeding behaviour of the European hedgehog (*Erinaceus europaeus *L.) in a New Zealand pasture* University of Canterbury]. https://researcharchive.lincoln.ac.nz/handle/10182/1825

Campbell, P. A. (1975). Feeding rhythms of caged hedgehogs (*Erinaceus europaeus* L.). *Proceedings (New Zealand Ecological Society), 22*, 14–18. http://www.jstor.org/stable/24064276

Citartan, M., Tang, T. H., Tan, C., Hoe, C. H., Saini, R., & Tominaga, J. (2012, 04/01). Asymmetric PCR for good quality ssDNA generation towards DNA aptamer production. *Songklanakarin Journal of Science and Technology, 34*, 125–131.

Deagle, B. E., Tollit, D. J., Jarman, S. N., Hindell, M. A., Trites, A. W., & Gales, N. J. (2005). Molecular scatology as a tool to study diet: analysis of prey DNA in scats from captive Steller sea lions. *Molecular Ecology, 14*(6), 1831–1842. https://doi.org/https://doi.org/10.1111/j.1365-294X.2005.02531.x

Dickman, C. R., & Huang, C. (1988). The reliability of fecal analysis as a method for determining the diet of insectivorous mammals. *Journal of Mammalogy, 69*(1), 108–113. https://doi.org/10.2307/1381753

Dierenfeld, E. S. (2009). Feeding behavior and nutrition of the african pygmy hedgehog (*Atelerix albiventris*). *Veterinary Clinics of North America: Exotic Animal Practice, 12*(2), 335–337. https://doi.org/https://doi.org/10.1016/j.cvex.2009.01.006

Duncan, R. P., Holland, E. P., Pech, R. P., Barron, M., Nugent, G., & Parkes, J. P. (2011). The relationship between possum density and browse damage on kamahi in New Zealand forests. *Austral Ecology, 36*(7), 858–869. https://doi.org/https://doi.org/10.1111/j.1442-9993.2010.02229.x

Egeter, B., Bishop, P. J., & Robertson, B. C. (2015). Detecting frogs as prey in the diets of introduced mammals: a comparison between morphological and DNA-based diet analyses. *Molecular Ecology Resources, 15*(2), 306–316. https://doi.org/https://doi.org/10.1111/1755-0998.12309

Egeter, B. J. C. (2014). *Detecting Frogs as Prey in the Diets of Introduced Mammals* [PhD Thesis] University of Otago. http://hdl.handle.net/10523/4688

Emami-Khoyi, A. (2015). *Population and diet of the New Zealand fur seal (*Arctocephalus forsteri*): molecular approaches* [PhD Thesis] Lincoln University. https://hdl.handle.net/10182/6758

Emami-Khoyi, A., Hartley, D. A., Paterson, A. M., Boren, L. J., Cruickshank, R. H., Ross, J. G., Murphy, E. C., & Else, T.-A. (2016). Identifying prey items from New Zealand fur seal (*Arctocephalus forsteri*) faeces using massive parallel sequencing. *Conservation Genetics Resources, 8*(3), 343–352. https://doi.org/10.1007/s12686-016-0560-9

Fountain, E., Pugh, A., & Bowie, M. H. (2013). *What do predators eat for supper? Burkes Pass Scenic Reserve predator stomach content analysis for 2010–2011*. https://researcharchive.lincoln.ac.nz/handle/10182/6814

Freeman, B. G., Scholer, M. N., Ruiz-Gutierrez, V., & Fitzpatrick, J. W. (2018). Climate change causes upslope shifts and mountaintop extirpations in a tropical bird community. *Proceedings of the National Academy of Sciences, 115*(47), 11982–11987. https://doi.org/10.1073/pnas.1804224115

Gonçalves, J., Marks, C. A., Obendorf, D., Amorim, A., & Pereira, F. (2014). The risks of using “species-specific” PCR assays in wildlife research: The case of red fox (*Vulpes vulpes*) identification in Tasmania. *Forensic Science International: Genetics, 11*, 9–11. https://doi.org/https://doi.org/10.1016/j.fsigen.2014.03.009

Gosselin, E. N., Lonsinger, R. C., & Waits, L. P. (2017). Comparing morphological and molecular diet analyses and fecal DNA sampling protocols for a terrestrial carnivore. *Wildlife Society Bulletin, 41*(2), 362–369. https://doi.org/https://doi.org/10.1002/wsb.749

Jones, C., Moss, K., & Sanders, M. (2005). Diet of hedgehogs (*Erinaceus europaeus*) in the upper Waitaki Basin, New Zealand: Implications for conservation. *New Zealand Journal of Ecology, 29*(1), 29–35. https://newzealandecology.org/nzje/2244

Jones, C., & Norbury, G. (2011). Feeding selectivity of introduced hedgehogs *Erinaceus europaeus* in a dryland habitat, South Island, New Zealand. *Acta Theriologica, 56*(1), 45–51. https://doi.org/10.1007/s13364-010-0009-6

Lear, G., Dickie, I., Banks, J., Boyer, S., Buckley, H., Buckley, T., Cruickshank, R., Dopheide, A., Handley, K., Hermans, S., Kamke, J., Lee, C., MacDiarmid, R., Morales, S., Orlovich, D., Smissen, R., Wood, J., & Holdaway, R. (2017). Methods for the extraction, storage, amplification and sequencing of DNA from environmental samples. *New Zealand Journal of Ecology*. https://doi.org/10.20417/nzjecol.42.9

Marris, J. W. M. (2000). The beetle (Coleoptera) fauna of the Antipodes Islands, with comments on the impact of mice and an annotated checklist of the insect and arachnid fauna. *Journal of the Royal Society of New Zealand, 30*(2), 169–195. https://doi.org/10.1080/03014223.2000.9517616

McKenna, D. D., Shin, S., Ahrens, D., Balke, M., Beza-Beza, C., Clarke, D. J., Donath, A., Escalona, H. E., Friedrich, F., Letsch, H., Liu, S., Maddison, D., Mayer, C., Misof, B., Murin, P. J., Niehuis, O., Peters, R. S., Podsiadlowski, L., Pohl, H., Scully, E. D., Yan, E. V., Zhou, X., Ślipiński, A., & Beutel, R. G. (2019). The evolution and genomic basis of beetle diversity. *Proceedings of the*

National Academy of Sciences U S A, 116(49), 24729–24737. https://doi.org/10.1073/pnas.1909655116

Park, K. (2004). Assessment and management of invasive alien predators. *Ecology and Society, 9*(2), 12. https://doi.org/10.5751/ES-01208-090212

Rosel, P. E., & Kocher, T. D. (2002). DNA-based identification of larval cod in stomach contents of predatory fishes. *Journal of Experimental Marine Biology and Ecology, 267*(1), 75–88. https://doi.org/https://doi.org/10.1016/S0022-0981(01)00359-8

Schneyer, N. (2002). *Effects of avian predation and habitat degradation on the population dynamics of the jewelled gecko (*Naultinus Gemmeus*) from the Every Scientific Reserve, Otago Peninsula, New Zealand (Thesis, Master of Science)* University of Otago. http://hdl.handle.net/10523/8416

Tabarzad, M., Kazemi, B., Vahidi, H., Aboofazeli, R., Shahhosseini, S., & Nafissi-Varcheh, N. (2014). Challenges to Design and Develop of DNA Aptamers for Protein Targets. I. Optimization of Asymmetric PCR for Generation of a Single Stranded DNA Library. *Iranian journal of pharmaceutical research, 13*, 133–141. https://www.researchgate.net/publication/261445475

Thomsen, P. F., & Willerslev, E. (2015). Environmental DNA – An emerging tool in conservation for monitoring past and present biodiversity. *Biological Conservation, 183*, 4–18. https://doi.org/https://doi.org/10.1016/j.biocon.2014.11.019

Vink, C., McNeill, M., Winder, L., Kean, J., & Phillips, C. (2011). PCR analyses of gut contents of pasture arthropods. In *Paddock to PCR - Demystifying molecular biology for practical plant protection* (pp. 125–134). New Zealand Plant Protection Society.

Vink, C. J., & Kean, J. M. (2013). PCR gut analysis reveals that *Tenuiphantes tenuis* (Araneae: Linyphiidae) is a potentially significant predator of Argentine stem weevil, *Listronotus bonariensis* (Coleoptera: Curculionidae), in New Zealand pastures. *New Zealand Journal of Zoology, 40*(4), 304–313. https://doi.org/10.1080/03014223.2013.794847

Wu, D. Y., Ugozzoli, L., Pal, B. K., Qian, J., & Wallace, R. B. (1991). The effect of temperature and oligonucleotide primer length on the specificity and efficiency of amplification by the polymerase chain reaction. *DNA and cell biology, 10 3*, 233–238.

Chapter 3

Comparing the quality of DNA in hedgehog's (*Erinaceus europaeus*) stomach and colon.

3.1 Introduction

The interactions between species in an ecosystem are important to understand because, among other things, they directly influence the management systems for conservation and pest control. Dietary analysis is a useful tool for identifying interactions between trophic levels and will aid with predicting the impact of an invasive species on native species, including competition, herbivory, and predation (Park 2004). Methods to analyse a species diet include direct observation (Fleurance et al. 2022), visual analysis of stomach content (Brockie 1958) and faeces (Dickman & Huang 1988), and DNA analysis of stomach contents (Vink et al. 2011) and faeces (Emami-Khoyi et al. 2016). Many factors potentially limit the success of each of these methods. Direct observations can introduce an observer bias based on experience levels (Fitzpatrick et al. 2009) but can record feeding events more reliably than video recording due to limitations, such as field of view, black and white picture, and poor focus (Tosi et al. 2006). Visual analysis of stomach and faecal contents is useful; however, hard body parts are more likely to survive digestion and be overrepresented in the samples (Deagle et al. 2005; Snider et al. 2021). DNA methods can increase the detection of prey in stomach contents and faeces of small mammals (Egeter et al. 2015), even after 16 hours of digestion in some species (Carreon-Martinez et al. 2011).

The use of dietary DNA analysis through PCR amplification (see Chapter 2) can show more depth of analysis with a much greater output of dietary items than visual observations (Emami-Khoyi et al. 2016) due to also detecting soft-bodied dietary items (Deagle et al. 2005). DNA approaches also have limitations. Some species have compounds that inhibit the amplification of dietary DNA from stomachs (Alikhani et al. 2019), such as bile salts in faeces, haem in blood, and urea in urine (Buckwalter et al. 2014). DNA in faeces may be degraded by digestion (Deagle et al. 2005), environmental exposure (Woodruff et al. 2015), and substrate contamination (McInnes et al. 2017).

Collecting content from stomachs will reduce degradation of dietary DNA, relative to faeces, as it spends less time in the digestion process (Snider et al. 2021). There are now solutions to inhibitors, such as adding BSA (bovine serum albumin) to a PCR reaction mix (Juen & Traugott 2006). Faeces are also still frequently used as it is a non-invasive way of analysing diet without having to capture or

euthanise target animals (Snider et al. 2021). When comparing stomach and faecal contents, although less DNA is shown in the faeces, the proportion of prey detected remained largely unaffected, and that the two methods are comparable (Snider et al. 2021).

When a study has many different sample sites and potentially limiting factors, it is important to maximise the effectiveness of the sample and obtain the most reliable output of dietary DNA. For this chapter, we compared the DNA quality of stomach and colon content from the same hedgehog. We mimicked faecal and stomach pairs that have been compared in the literature (Egeter et al. 2015; Snider et al. 2021) by using colon content to supplement the faeces. Using the stomach and colon content from the same animal ensures each sample is independent (Robeson et al. 2018). I predict that the stomach content will show stronger DNA output given that colon contents have spent longer in the digestion process and have had more opportunity for the DNA to degrade.

3.2 Objective

3.2.1 Compare the DNA output in the stomach and colon content.

The DNA in the stomachs of hedgehogs was predicted to be less degraded than DNA in the colon. It was hoped that this could be visualised as longer fragments by gel electrophoresis.

3.3 Methods

3.3.1 Collection of Hedgehogs

Hedgehogs were collected from across Kaitorete Spit (-43.82°, 172.60°) from a range of habitats, including, pasture grassland, tussock grassland on dunes and lakeside shrubby habitat. The collection was made during pre-existing live trapping by the Department of Conservation (DOC) and Pest Free Banks Peninsula (PFBP) and euthanised according to DOC and PFBP protocol using a knife to the back of the head before being frozen. These hedgehogs were also used in Chapter 5, see Chapter 4 for details on the site.

3.3.2 Dissection and collection of stomach and colon content

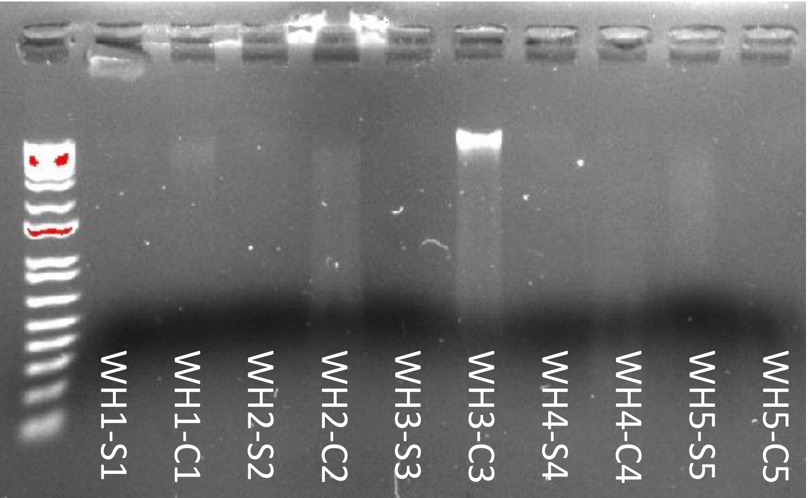
Ten hedgehogs were dissected with an incision along the abdomen through both skin and muscle layers to expose the abdominal cavity. Stomachs were then removed by cutting at the terminal end of the oesophagus (cardia) and before the beginning of the small intestine (pylorus). Once removed, the stomach was cut along the short side (lesser curvature) and folded inside out. The stomach contents were scrapped off the stomach lining into a collection vial with a clean scalpel, which included all visible fragments and mucus. Colon content was collected from about 70 mm along the colon and large intestine. The colon was exposed by breaking the pelvis and cutting the colon and anus away from the rest of the body, but not severing from the large intestine. The contents were squeezed out of the colon through the anus into a collection vial, mimicking natural defecation. All utensils were cleaned with hot water between individuals. After dissection, samples were frozen at -20 °C before further analysis. The remaining carcasses were frozen separately.

3.3.3 DNA analysis.

For each dissected hedgehog there was a paired sample of stomach and colon content. The five heaviest sets were used for DNA analysis and another two sets were provided as spare samples for section 5.3.2. The remaining three sets lacked sufficient content volume (<1.5 g) for DNA extraction. The DNA was extracted using a QIAGEN DNeasy Power Max soil kit as outlined in Chapter 2. In the case when the total faecal or colon control weighted more than 10 g, a sub-sample of <10 g was taken after mixing the content with a sterile pipette tip. Raw DNA was stained with BIOLINE 5x DNA loading buffer (blue) and run on a 1% Agarose gel dyed with INtRON RedSafe™ nucleic acid staining solution for 30 minutes at 100 volts to visualise the amplified DNA. The gel was visualised in a Bio Rad GelDoc Go Gel Imaging System. One microlitre of each raw DNA sample was also run through a Nanodrop 2000/2000c Spectrophotometer and the output was recorded (Table 3-1).

3.4 Results

The wild hedgehogs gel electrophoresis output was more visible from the colon content compared to the stomach content. Colon content was visible in 4/5 samples while the stomach content was visible in only 1/5 samples (Figure 3-1).



WH = Wild Hedgehog
S = Stomach
C= Colon

Figure 3-1. Crude DNA comparing stomach and colon DNA on gel electrophoresis.

The nanodrop output showed no relationship to the gel electrophoresis output with variable quantity and quality of samples (Table 3-1). Quantity ranged from 14.3–34.3 ng/µL for colon and 1.2–36.7 ng/µL for stomach. The quality ranged from 1.22–1.63 A260/A280 and 1.34–1.69 A260/A280 for the stomach and colon contents, respectively.

Table 3-1. Sample weight and Nanodrop output from raw DNA samples of hedgehog stomach and colon content.

Code	Data type	Quantity ng/µL	Quality A260/A280	Weight g
Target Range		*>10*	*1.7-1.8*	*10*
WH1-C1	Colon	14.3	1.34	4.63
WH2-C2	Colon	25.0	1.69	2.87
WH3-C3	Colon	26.9	1.53	3.65
WH4-C4	Colon	15.9	1.63	3.5
WH5-C5	Colon	34.3	1.36	4.33
WH1-S1	Stomach	1.2	1.63	4.31
WH2-S2	Stomach	1.6	1.48	4.29

WH3-S3	Stomach	36.7	1.22	9.73
WH4-S4	Stomach	1.8	1.36	1.81
WH5-S5	Stomach	6.6	1.60	9.74

3.5 Discussion

I compared the DNA output between the stomach and colon content of hedgehogs. Longer fragments of DNA were extracted from colon contents than from the stomach contents. This result was the opposite of what was expected based on the known impacts of digestion (Deagle et al. 2005; Snider et al. 2021). I predicted that the DNA from the stomach content would have brighter and longer bands in the gel than the DNA from the colon content, given that the contents have spent longer in the digestion process and have had more opportunity for the DNA to degrade. This prediction was based on Paquin and Vink (2009) who investigated the impact of non-destructive DNA extraction methods for sequencing individual species. All extraction methods from Paquin and Vink (2009) showed visible smears of DNA on a 0.8% agarose gel.

These results suggest that something in the stomach might inhibit DNA detection, DNA extraction, DNA staining on gels, or any combination of these. Hedgehog cells sloughed from the colon and small intestine during digestion may have resulted in longer DNA fragments. These fragments are unlikely to be present in the stomach samples and may have contributed to the observed difference. Further testing with a different gel staining method, extraction method, or amplifying the DNA with PCR may be required to provide a reliable output from stomach samples. These samples were not put through a PCR protocol because this was intended to be an easy method with minimal steps. If a PCR protocol were to be followed, the addition of BSA (bovine serum albumin) to the PCR reaction mix (Juen & Traugott 2006) might be required to overcome any gastric DNA inhibition.

The extracted DNA samples were analysed with a Nanodrop spectrophotometer that uses light to determine the quality of samples and quantity of DNA present. The nanodrop showed a very low quantity for the stomach (1.2–6.6 ng/µL) except for one sample with a higher quantity of 36.7 ng/µL. This low quantity could contribute to the poor visualisation of DNA in the gels. Low concentrations of DNA below 20 ng/µL are considered unreliable due to variable concentration readings for the same sample and several replicates should be taken or a more sensitive method may be needed (e.g., Qubit) (Koetsier & Cantor 2019). The quality of DNA from the stomach and colon were both below 1.8 A260/A280. This is considered a low-quality reading and indicates that there could be protein

contamination which may affect downstream applications and it should be considered to re-extract samples (Koetsier & Cantor 2019).

The hedgehog has an acidic stomach with a pH between 4.5 and 4.8 when there is food in it (Morris & Steel 1967). The pH level of a sample can significantly alter the A260/A280 ratio with an acidic pH level having a decreasing effect (Wilfinger et al. 1997). To counter this effect, a buffering agent (e.g., Na_2HPO_4) may be added to balance the pH level (Wilfinger et al. 1997). The pH level of the samples in the present study was not measured but should be considered for future analysis if it becomes a significant limiting factor.

I would recommend following the nanodrop test with a fragment analyser that could show the lengths of the raw DNA fragments and the possible impact of digestion. Colon contents should have shorter fragments due to greater exposure to mechanical and chemical breakdown during digestion (Snider et al. 2021). Digested material has shorter fragments of DNA, about 300 base pairs long. Shorter fragments will have a negative impact when a generic primer may focus on larger fragments of DNA and not detect the digested material, therefore, specific primers may need to be designed.

3.6 Conclusion

While the protocol in this study did not show a result to compare the stomach and colon content of hedgehogs, there were important factors to consider. The DNA extraction and visualisation of stomach contents may have been limited by DNA inhibitory compounds that need to be overcome and then this method may be successful. There is also further analysis that could be done to define the quality of the extracted DNA such as a fragment analyser or Qubit. Any variation in the quality of DNA from the different sources is important to distinguish where to sample from for the most reliable output of dietary DNA. Regardless of this protocol having problems, the study did show that, in principle, dietary DNA can be sampled from the hedgehog digestive system. The dietary information is important to understand ecological relationships between predators and prey and contributes to efficient conservation and pest control management programmes.

3.7 References

Alikhani, M., Shafaie, E., Mirabzadeh Ardakani, E., Esmaeili, M., Saberi, S., Hatefi, M., & Mohammadi, M. (2019). the inhibitory effect of mouse gastric dna on amplification of *Helicobacter pylori* genomic DNA in quantitative PCR. *Iranian Biomedical Journal, 23*(4), 297–302. https://doi.org/10.29252/.23.4.297

Brockie, B. (1958). *The ecology of the hedgehog (*Erinaceus europaeus L.*) in Wellington, New Zealand : [a thesis] submitted for the degree of Master of Science in Zoology at the Victoria University of Wellington* https://mro.massey.ac.nz/server/api/core/bitstreams/594fd3e7-cc8f-41e8-86cb-6f2b49bad97b/content

Buckwalter, S. P., Sloan, L. M., Cunningham, S. A., Espy, M. J., Uhl, J. R., Jones, M. F., Vetter, E. A., Mandrekar, J., Cockerill, F. R., 3rd, Pritt, B. S., Patel, R., & Wengenack, N. L. (2014). Inhibition controls for qualitative real-time PCR assays: are they necessary for all specimen matrices? *J Clin Microbiol, 52*(6), 2139–2143. https://doi.org/10.1128/jcm.03389-13

Carreon-Martinez, L., Johnson, T. B., Ludsin, S. A., & Heath, D. D. (2011). Utilization of stomach content DNA to determine diet diversity in piscivorous fishes. *J Fish Biol, 78*(4), 1170–1182. https://doi.org/10.1111/j.1095-8649.2011.02925.x

Deagle, B. E., Tollit, D. J., Jarman, S. N., Hindell, M. A., Trites, A. W., & Gales, N. J. (2005). Molecular scatology as a tool to study diet: analysis of prey DNA in scats from captive Steller sea lions. *Molecular Ecology, 14*(6), 1831–1842. https://doi.org/https://doi.org/10.1111/j.1365-294X.2005.02531.x

Dickman, C. R., & Huang, C. (1988). The reliability of fecal analysis as a method for determining the diet of insectivorous mammals. *Journal of Mammalogy, 69*(1), 108–113. https://doi.org/10.2307/1381753

Egeter, B., Bishop, P. J., & Robertson, B. C. (2015). Detecting frogs as prey in the diets of introduced mammals: a comparison between morphological and DNA-based diet analyses. *Molecular Ecology Resources, 15*(2), 306–316. https://doi.org/https://doi.org/10.1111/1755-0998.12309

Emami-Khoyi, A., Hartley, D. A., Paterson, A. M., Boren, L. J., Cruickshank, R. H., Ross, J. G., Murphy, E. C., & Else, T.-A. (2016). Identifying prey items from New Zealand fur seal (*Arctocephalus forsteri*) faeces using massive parallel sequencing. *Conservation Genetics Resources, 8*(3), 343–352. https://doi.org/10.1007/s12686-016-0560-9

Fitzpatrick, M. C., Preisser, E. L., Ellison, A. M., & Elkinton, J. S. (2009). Observer bias and the detection of low-density populations. *Ecological Applications, 19*(7), 1673–1679. https://doi.org/https://doi.org/10.1890/09-0265.1

Fleurance, G., Rossignol, N., & Dumont, B. (2022). Diurnal observations of feeding choices in grazing horses correctly predict their daily diet composition. *Applied Animal Behaviour Science, 252*, 105652. https://doi.org/https://doi.org/10.1016/j.applanim.2022.105652

Juen, A., & Traugott, M. (2006). Amplification facilitators and multiplex PCR: Tools to overcome PCR-inhibition in DNA-gut-content analysis of soil-living invertebrates. *Soil Biology and Biochemistry, 38*(7), 1872–1879. https://doi.org/https://doi.org/10.1016/j.soilbio.2005.11.034

Koetsier, G., & Cantor, E. (2019). *A Practical Guide to Analyzing Nucleic Acid Concentration and Purity with Microvolume Spectrophotometers*. New England Biolabs. https://www.neb.com/en/-/media/nebus/files/application-notes/technote_mvs_analysis_of_nucleic_acid_concentration_and_purity.pdf?rev=c24cea043416420d84fb6bf7b554dbbb

McInnes, J. C., Alderman, R., Deagle, B. E., Lea, M.-A., Raymond, B., & Jarman, S. N. (2017). Optimised scat collection protocols for dietary DNA metabarcoding in vertebrates. *Methods in Ecology and Evolution, 8*(2), 192–202. https://doi.org/https://doi.org/10.1111/2041-210X.12677

Morris, B., & Steel, E. D. (1967). Gastric and duodenal differentiation in *Erinaceus europaeus* and its relationship to antibody absorption. *Journal of Zoology, 152*(3), 257–267. https://doi.org/https://doi.org/10.1111/j.1469-7998.1967.tb01642.x

Paquin, P., & Vink, C. J. (2009). Testing compatibility between molecular and morphological techniques for arthropod systematics: a minimally destructive DNA extraction method that preserves morphological integrity, and the effect of lactic acid on DNA quality. *Journal of Insect Conservation, 13*(4), 453–457. https://doi.org/10.1007/s10841-008-9183-0

Park, K. (2004). Assessment and management of invasive alien predators. *Ecology and Society, 9*. https://doi.org/10.5751/ES-01208-090212

Robeson, M. S., II, Khanipov, K., Golovko, G., Wisely, S. M., White, M. D., Bodenchuck, M., Smyser, T. J., Fofanov, Y., Fierer, N., & Piaggio, A. J. (2018). Assessing the utility of metabarcoding for diet analyses of the omnivorous wild pig (*Sus scrofa*). *Ecology and Evolution, 8*(1), 185–196. https://doi.org/https://doi.org/10.1002/ece3.3638

Snider, A. M., Bonisoli-Alquati, A., Pérez-Umphrey, A. A., Stouffer, P. C., & Taylor, S. S. (2021). Metabarcoding of stomach contents and fecal samples provide similar insights about Seaside Sparrow diet. *Ornithological Applications, 124*(1), 1–8, 8. https://doi.org/10.1093/ornithapp/duab060

Tosi, M. V., Ferrante, V., Mattiello, S., Canali, E., & Verga, M. (2006). Comparison of video and direct observation methods for measuring oral behaviour in veal calves. *Italian Journal of Animal Science, 5*(1), 19–27. https://doi.org/10.4081/ijas.2006.19

Vink, C., McNeill, M., Winder, L., Kean, J., & Phillips, C. (2011). PCR analyses of gut contents of pasture arthropods. In *Paddock to PCR - Demystifying molecular biology for practical plant protection* (pp. 125–134). New Zealand Plant Protection Society.

Wilfinger, W. W., Mackey, K., & Chomczynski, P. (1997). Effect of pH and ionic strength on the spectrophotometric assessment of nucleic acid purity. *Biotechniques, 22*(3), 474–481. https://doi.org/10.2144/97223st01

Woodruff, S. P., Johnson, T. R., & Waits, L. P. (2015). Evaluating the interaction of faecal pellet deposition rates and DNA degradation rates to optimize sampling design for DNA-based mark–recapture analysis of Sonoran pronghorn. *Molecular Ecology Resources, 15*(4), 843–854. https://doi.org/https://doi.org/10.1111/1755-0998.12362

Chapter 4

A DNA reference library of invertebrates from Kaitorete Spit.

4.1 Introduction

Biodiversity monitoring is important to track ecosystem changes, such as population fluctuations (Saunders et al. 2019), breeding success (Bell 2017), invasive species spread (Christopher & Cameron 2012), and habitat loss (Bogich et al. 2012). Understanding these changes helps to determine what conservation efforts need to be implemented to preserve ecosystem functions (Cardinale et al. 2012).

To achieve effective monitoring, there needs to be a standardised method for identifying species. Traditional identification methods use morphological features to follow a dichotomous key (Specchia et al. 2020). Current estimates for the number of animal species are about 7.8 million, of which about 1.2 million are described, leaving 6.6 million species unidentified (Mora et al. 2011; Wiens 2023). Arthropods are the largest phylum of animals with an estimated 6.8 million species (Mora et al. 2011; Stork et al. 2015), with 1.2 million species described (Stork 2018). The remaining number of species are undescribed, which makes the identification of species in many parts of the world (including New Zealand) very challenging, if not impossible.

To improve the speed and accuracy of species identification, DNA can be used (deWaard et al. 2019). Each species has unique DNA that can be databased, known as a barcode (Deagle et al. 2019), most commonly from the mitochondrial region cytochrome oxidase I gene (COI) (Specchia et al. 2020). The GenBank database has molecular data matched to over 160,000 organisms with their classification and nomenclature, representing about 10% of currently described species (https://www.ncbi.nlm.nih.gov/). There are also 291,000 arthropod species with barcodes on the Barcode of Life Data System (BOLD Systems; https://www.boldsystems.org/index.php).

A species barcode can be used to analyse a sample for a single species using a short targeting fragment of DNA called a primer pair (Gonçalves et al. 2014). All species in a sample can be separated using high throughput sequencing (HTS) and metabarcoding to match the unique DNA sequences to known barcodes (Emami-Khoyi et al. 2016; Thomsen & Willerslev 2015). Environmental DNA (eDNA) is an effective sampling approach that collects all DNA present from an environmental sample, such as soil, sediment and water (Thomsen & Willerslev 2015). The advantage of using molecular identification over morphology is being able to identify species from

both adult and juvenile forms of species, which is often difficult to do with morphology alone (Specchia et al. 2020); however, molecular methods cannot distinguish between lift stages (Thomsen & Willerslev 2015).

The success of identification using DNA is highly dependent on having a reliable and complete reference barcode library to match to unknown DNA in samples (Specchia et al. 2020; Thomsen & Willerslev 2015). Specchia et al. (2020) emphasise that scientific efforts should be targeted towards forming complete DNA libraries to lead to more efficient identification when assessing biodiversity. Ideally, a reference library is created to include all the species present from a location to prevent misidentification from poor DNA matches. The process of metabarcoding will match a sequence of DNA with the closest species match from a given database and give it a percentage grade (Verkuil et al. 2022). If there are no species from the target area included in the reference library, apparent matches are unlikely to be correct; however, incorrect species matches can indicate appropriate higher taxonomic level identifications if similar species have been barcoded previously.

We created a reference library for Kaitorete Spit to support the high throughput sequencing in Chapter 5. The library was created to represent the unique invertebrate fauna on Kaitorete Spit, containing many species endemic to Kaitorete Spit or on similar coastal rocky and sandy dune habitats. These species include the Kaitorete Spit endemic Kupe’s moth (*Kupea electilis*) (Gaskin 1975), the New Zealand endemic katipō spider (*Latrodectus katipo*) (Hetherington & Wilson 2014), and the large sand scarab (*Pericoptus truncatus* (Fabricius, 1775)) (Ratcliffe & Orozco 2009).

4.2 Objective

Collect, curate, barcode, and database invertebrates from Kaitorete Spit to create a DNA reference library to support high throughput sequencing of the prey items present in hedgehog faeces.

4.3 Methods

4.3.1 Study Site

This study focussed on invertebrates from Kaitorete Reserve, managed by the Christchurch City Council. The reserve is located at the Birdlings Flat end of the spit on the Lake Ellesmere side (Figure 4-1). See Chapter 1 for information on the habitat and formation of Kaitorete Spit.

Figure 4-1. Map of Kaitorete Spit with Kaitorete Reserve in white. Retrieved from Google Earth.

4.3.2 Collection of Species

Invertebrates were collected from Kaitorete Reserve on Kaitorete Spit during February 2023 with pitfall traps, sweep netting and hand collecting. Eight pitfall traps were set up at 200–500 m intervals along a transect line that Christchurch City Council established for ongoing invertebrate monitoring (Figure 4-2). Pitfall traps consisted of a plastic cup with a diameter of 85 mm placed into a hole in the ground, so that the top of the cup was flush with the surface. The cups were filled with a 100 mL mix of water and ethylene glycol to kill and preserve the samples. Pitfall traps were set up on 20 February 2023 and collected on 27 February 2023. Opportunistic sweep netting occurred in the reserve and a neighbouring grazed paddock, while hand collection occurred primarily by rolling longs and with opportunistic interceptions. All specimens collected were labelled with date and location.

Figure 4-2. The layout of pitfall traps at Kaitorete Reserve that was established by the Christchurch City Council. Retrieved from Google Earth.

4.3.3 Curation and Identification

Specimens were identified to family, genus, or species where possible using morphology features using the following guides, Coleoptera (Klimaszewski & Watt 1997; Larochelle & Larivière 2007; Watt 1992), Hemiptera (Larivière et al. 2010; Larivière 1995), Araneae (Paquin et al. 2010; Vink 2002), Hymenoptera (Donovan 2007; Ward 2024), and general specimens (iNaturalist NZ n.d.). Dorsal, lateral, and ventral photos were taken using a Nikon DS-Ril camera attached to a Nikon stereomicroscope. Voucher specimens were curated following guidelines from Walker and Crosby (1988) and catalogued in the Lincoln University Entomology Research Collection, New Zealand.

4.3.4 Barcoding DNA

Fifty specimens were prepared for sequencing by removing one or two legs for smaller specimens < 5 mm. These legs were then stored in 90% ethanol for storage and transport to the Centre for Biodiversity Genomics, University of Guelph for further analysis. Specimens were chosen based on their uniqueness/endemism to Kaitorete Spit and the absence of DNA sequence on GenBank. The ratio of specimens chosen from each order was based on the proportion found in observations on iNaturalist (https://inaturalist.nz/observations ?iconic_taxa=Mollusca,Arachnida,Insecta&place_id=68103&subview=table) from Kaitorete Spit (Table 4-2). DNA extraction was performed by using solid-phase reversible immobilization (SPRI) and stored at -20 °C until use (Hebert et al. 2018). See Hebert et al. (2023) for details on the laboratory

protocols for sequencing a standard COI marker by multiplexing pooled PCR samples using an Oxford Nanopore Technologies Flongle 10.4.1 Flow Cell on a MinION sequencer.

4.3.5 BOLD systems

Sequences were uploaded onto the BOLD systems webpage for access and analysis (Table 4-1). All 50 specimens were uploaded to BOLD systems with photos (dorsal, lateral, and ventral), collection location, morphological identification, and DNA sequence if successful.

Table 4-1. Barcode of Life Database pages for all sequenced specimens. The specimen page includes collection information, photographs, and taxonomy (Figure 4-3). Morphological ID is from 4.3.3. The sequence page includes the illustrative barcode and nucleotide sequence (Figure 4-4). The BIN number collates all records of the same sequence into one page (Figure 4-5). Each species contains hyperlinks for the specimen page, sequence page and BIN number.

Specimen Page	Morphological ID	Sequence Page	BIN Number
NZ750_2023	*Pericoptus* sp.	LMOUY390-23	BOLD:ADO6466
NZ751_2023	*Mimopeus* sp	LMOUY389-23	NA
NZ752_2023	*Moriomorphini* sp	LMOUY388-23	BOLD:ABW2846
NZ753_2023	*Mimopeus sp*	LMOUY387-23	NA
NZ754_2023	*Haplanister crypticus*	LMOUY386-23	BOLD:ACP3516
NZ755_2023	*Anthicus sp*	LMOUY385-23	BOLD:AEU4186
NZ756_2023	Curculionidae	LMOUY384-23	BOLD:AFE0080
NZ757_2023	Curculionidae	LMOUY383-23	BOLD:ACI2251
NZ758_2023	*Ataenius picinus*	LMOUY382-23	BOLD:AFC3591
NZ759_2023	Staphylinidae	LMOUY381-23	NA
NZ760_2023	Muscidea	LMOUY399-23	BOLD:AFC8776
NZ761_2023	*Calliphora sp*	LMOUY398-23	BOLD:AAB6579
NZ762_2023	Muscoidea	LMOUY397-23	BOLD:AFC2380
NZ763_2023	*Anabarhynchus sp*	LMOUY396-23	BOLD:AFC8780
NZ764_2023	*Saropogon sp*	LMOUY395-23	BOLD:AFC7074
NZ765_2023	*Tetragnatha sp*	LMOUY394-23	BOLD:AFC4726
NZ766_2023	*Tetragnatha sp*	LMOUY393-23	BOLD:AFC4726
NZ767_2023	Araneoidea	LMOUY392-23	BOLD:AFC5367
NZ768_2023	*Anoteropsis hilaris*	LMOUY391-23	BOLD:ACM2356
NZ769_2023	*Anoteropsis litoralis*	LMOUY410-23	NA
NZ770_2023	*Acrossidius tasmaniae*	LMOUY409-23	BOLD:AAP9995

Specimen Page	Morphological ID	Sequence Page	BIN Number
NZ771_2023	*Moriomorphini sp*	LMOUY408-23	BOLD:ABW2846
NZ772_2023	*Sitona sp*	LMOUY406-23	BOLD:ABV3850
NZ773_2023	Staphylinidae	LMOUY407-23	BOLD:AFC7184
NZ774_2023	Staphylinidae	LMOUY405-23	BOLD:AFC7184
NZ775_2023	*Phalangium opilio*	LMOUY404-23	BOLD:AAI4346
NZ776_2023	Cicadellidae	LMOUY403-23	BOLD:AFD6947
NZ777_2023	Cicadellidae	LMOUY402-23	BOLD:AFE0781
NZ778_2023	*Cydnochoerus nigrosignatus*	LMOUY401-23	NA
NZ779_2023	*Nysius sp*	LMOUY400-23	NA
NZ780_2023	*Rhypodes anceps*	LMOUY419-23	BOLD:AFC8067
NZ781_2023	*Nabis sp*	LMOUY418-23	BOLD:AFB8334
NZ782_2023	*Lasioglossum sp*	LMOUY417-23	NA
NZ783_2023	*Chelaner antarcticus*	LMOUY416-23	BOLD:AFD4225
NZ784_2023	Curculionidae	LMOUY415-23	NA
NZ785_2023	*Scoparia sp*	LMOUY414-23	BOLD:ACP5949
NZ786_2023	*Scoparia sp*	LMOUY413-23	BOLD:ACP5949
NZ787_2023	*Eudonia sp*	LMOUY412-23	BOLD:AAA2673
NZ788_2023	*Eudonia sp*	LMOUY411-23	BOLD:ACP5949
NZ789_2023	*Scoparia sp*	NA	NA
NZ790_2023	*Eudonia sp*	LMOUY429-23	BOLD:ACP5949
NZ791_2023	*Eudonia sp*	LMOUY428-23	BOLD:AAA2673
NZ792_2023	*Scoparia sp*	LMOUY427-23	BOLD:ACP2188
NZ793_2023	*Orocrambus sp*	LMOUY426-23	BOLD:ACP6432
NZ794_2023	*Gnathaphanus melbournensis*	LMOUY425-23	NA
NZ795_2023	Geophilomorpha	LMOUY424-23	BOLD:AFC5917
NZ796_2023	Lithobiidae	LMOUY423-23	BOLD:ACS1508
NZ797_2023	*Bobilla sp*	LMOUY422-23	BOLD:ADT4395
NZ798_2023	*Phaulacridium marginale*	LMOUY420-23	BOLD:AFD0220
NZ799_2023	*Bobilla sp*	LMOUY421-23	BOLD:ADT4395

4.4 Results

From the 50 specimens that were collected and sequenced, 41 returned successful sequences (Table 4-2) identified to 22 families, 20 genera and 14 species, as many had no identification at a lower taxonomic level (Table 4-3).

Table 4-2. The number of each invertebrate order that was sequenced for the invertebrate library. See Table 4-1 for the full identification list.

Order	Number Sequenced	Successful Sequences
Coleoptera	17	12
Lepidoptera	9	9
Hemiptera	6	4
Araneae	5	4
Diptera	5	5
Orthoptera	3	3
Chilopoda	2	2
Hymenoptera	2	1
Opiliones	1	1
Total	**50**	**41**

Table 4-3. List of the Invertebrate library comparing the morphological identification and DNA identification showing the percentage match of the DNA matches to public databases. Grey rows indicate unsuccessful DNA sequencing and a green to yellow colouration shows the percentage match with 100% being green. Of the sequences in the DNA library, 25 had grades >99% (14 species, six genera, and five family level).

Sample ID	Morphological ID	Order	Family	Genus	Species	Grade % Match
NZ750_2023	*Pericoptus* sp.	Coleoptera	Scarabaeidae	*Pericoptus*	*X*	100
NZ751_2023	*Mimopeus sp*	Coleoptera				
NZ752_2023	*Moriomorphini* sp	Coleoptera	Carabidae	*Mecyclothorax*	*X*	99.64
NZ753_2023	*Mimopeus sp*	Coleoptera				
NZ754_2023	*Haplanister crypticus*	Coleoptera	Carabidae	*X*	*X*	100
NZ755_2023	*Anthicus sp*	Coleoptera	Anthicidae	*X*	*X*	100
NZ756_2023	Curculionidae	Coleoptera	Curculionidae	*X*	*X*	97.07
NZ757_2023	Curculionidae	Coleoptera	Curculionidae	*Listronotus*	*Listronotus bonariensis*	100
NZ758_2023	*Ataenius picinus*	Coleoptera	Scarabaeidae	*X*	*X*	89.14
NZ759_2023	Staphylinidae	Coleoptera				
NZ760_2023	Muscidea	Diptera	Muscidae	*X*	*X*	93.39
NZ761_2023	*Calliphora sp*	Diptera	Calliphoridae	*Calliphora*	*Calliphora vicina*	100
NZ762_2023	Muscoidea	Diptera	Muscidae	*X*	*X*	97.3
NZ763_2023	*Anabarhynchus sp*	Diptera	Therevidae	*Anabarhynchus*	*Anabarhynchus aureosericeus*	92.96
NZ764_2023	*Saropogon sp*	Diptera	Asilidae	*Saropogon*	*X*	91.9
NZ765_2023	*Tetragnatha sp*	Araneae	Tetragnathidae	*Tetragnatha*	*X*	100
NZ766_2023	*Tetragnatha sp*	Araneae	Tetragnathidae	*Tetragnatha*	*X*	100
NZ767_2023	Araneoidea	Araneae	Araneidae	*Cyclosa*	*Cyclosa mulmeinensis*	88.06
NZ768_2023	*Anoteropsis hilaris*	Araneae	Lycosidae	*Anoteropsis*	*Anoteropsis hilaris*	98.92
NZ769_2024	*Anoteropsis litoralis*	Araneae				
NZ770_2023	*Acrossidius tasmaniae*	Coleoptera	Scarabaeidae	*Acrossidius*	*Acrossidius tasmaniae*	100
NZ771_2023	*Moriomorphini sp*	Coleoptera	Carabidae	*Mecyclothorax*	*Mecyclothorax rufipennis*	99.64
NZ772_2023	*Sitona sp*	Coleoptera	Curculionidae	*Sitona*	*Sitona obsoletus*	100
NZ773_2023	Staphylinidae	Coleoptera	Staphylinidae	*X*	*X*	99.85
NZ774_2023	Staphylinidae	Coleoptera	Staphylinidae	*X*	*X*	99.85
NZ775_2023	*Phalangium opilio*	Opiliones	Phalangiidae	*Phalangium*	*Phalangium opilio*	100
NZ776_2023	Cicadellidae	Hemiptera	Cicadellidae	*X*	*X*	88.1
NZ777_2023	Cicadellidae	Hemiptera	Cicadellidae	*X*	*X*	86.8
NZ778_2023	*Cydnochoerus nigrosignatus*	Hemiptera				
NZ779_2023	*Nysius sp*	Hemiptera				

Sample ID	Morphological ID	Order	Family	Genus	Species	Grade % Match
NZ780_2023	*Rhypodes anceps*	Hemiptera	Lygaeinae	*X*	*X*	89.88
NZ781_2023	*Nabis sp*	Hemiptera	Nabidae	*Nabis*	*X*	97.09
NZ782_2023	*Lasioglossum sp*	Hymenoptera				
NZ783_2023	*Chelaner antarcticus*	Hymenoptera	Formicidae	*Chelaner*	*Chelaner antarcticus*	95.15
NZ784_2023	Curculionidae	Coleoptera				
NZ785_2023	*Scoparia sp*	Lepidoptera	Crambidae	*Eudonia*	*Eudonia submarginalis*	100
NZ786_2023	*Scoparia sp*	Lepidoptera	Crambidae	*Eudonia*	*Eudonia submarginalis*	100
NZ787_2023	*Eudonia sp*	Lepidoptera	Crambidae	*Achyra*	*Achyra affinitalis*	99.85
NZ788_2023	*Eudonia sp*	Lepidoptera	Crambidae	*Scoparia*	*Eudonia submarginalis*	99.85
NZ789_2023	*Scoparia sp*	Lepidoptera				
NZ790_2023	*Eudonia sp*	Lepidoptera	Crambidae	*Eudonia*	*Eudonia submarginalis*	100
NZ791_2023	*Eudonia sp*	Lepidoptera	Crambidae	*Achyra*	*Achyra affinitalis*	99.85
NZ792_2023	*Scoparia sp*	Lepidoptera	Crambidae	*Scoparia*	*Scoparia chalicodes*	100
NZ793_2023	*Orocrambus sp*	Lepidoptera	Crambidae	*Orocrambus*	*Orocrambus vitellus*	100
NZ794_2023	*Gnathaphanus melbournensis*	Lepidoptera				
NZ795_2023	Geophilomorpha	Chilopoda	Geophilidae	*Stenotaenia*	*X*	81.87
NZ796_2023	Lithobiidae	Chilopoda	Henicopidae	*X*	*X*	100
NZ797_2023	*Bobilla sp*	Orthoptera	Trigonidiidae	*Bobilla*	*X*	99.54
NZ798_2023	*Phaulacridium marginale*	Orthoptera	Acrididae	*X*	*X*	95.38
NZ799_2023	*Bobilla sp*	Orthoptera	Trigonidiidae	*Bobilla*	*X*	99.54

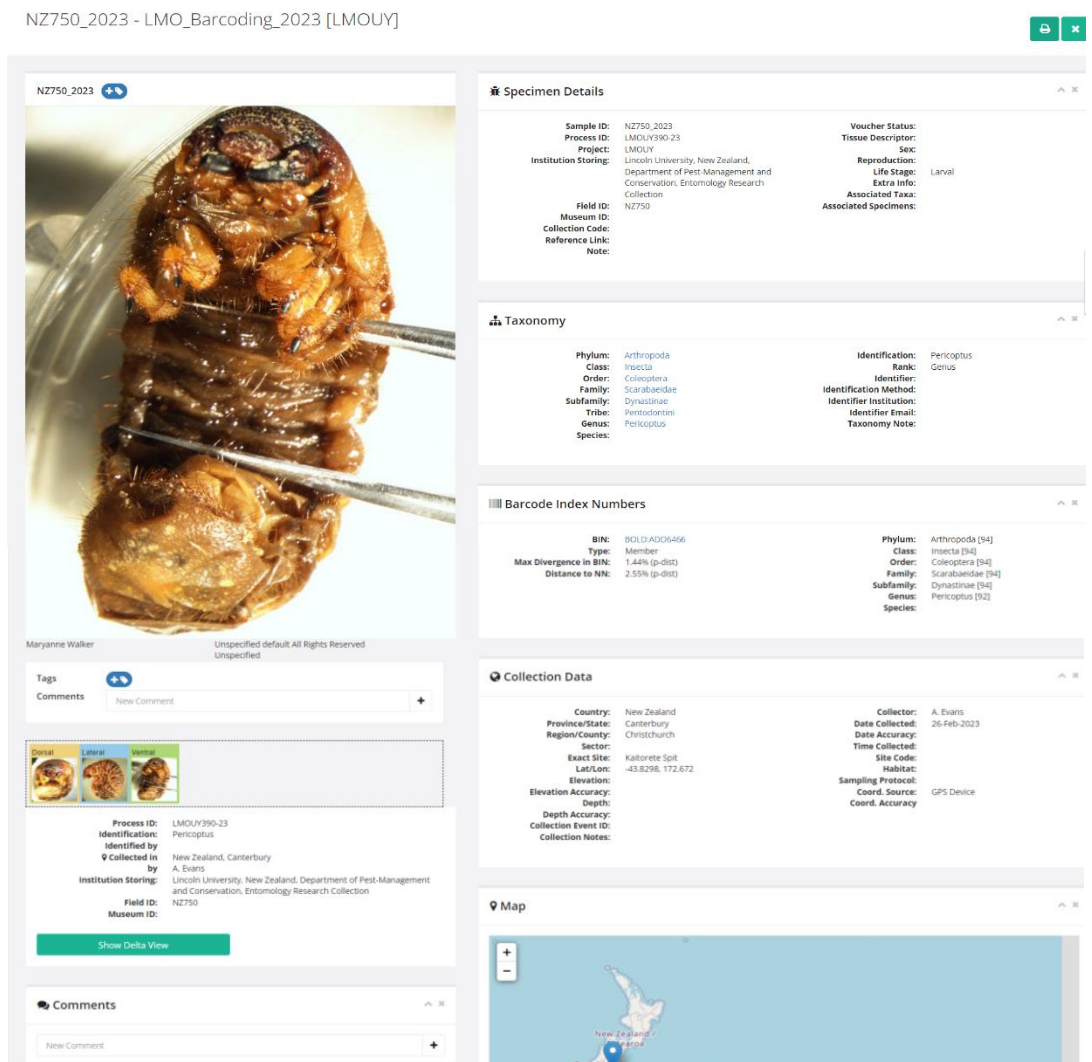


Figure 4-3. Example of a BOLD specimen page for sample NZ750_2023 - *Pericoptus* sp.

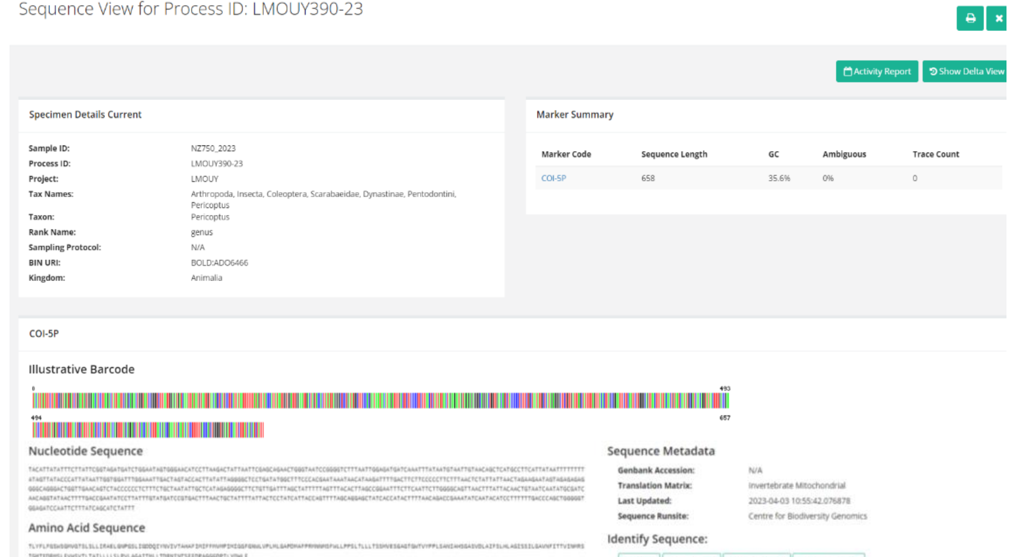


Figure 4-4. Example BOLD sequence page for sample NZ750_2023 - *Pericoptus* sp.

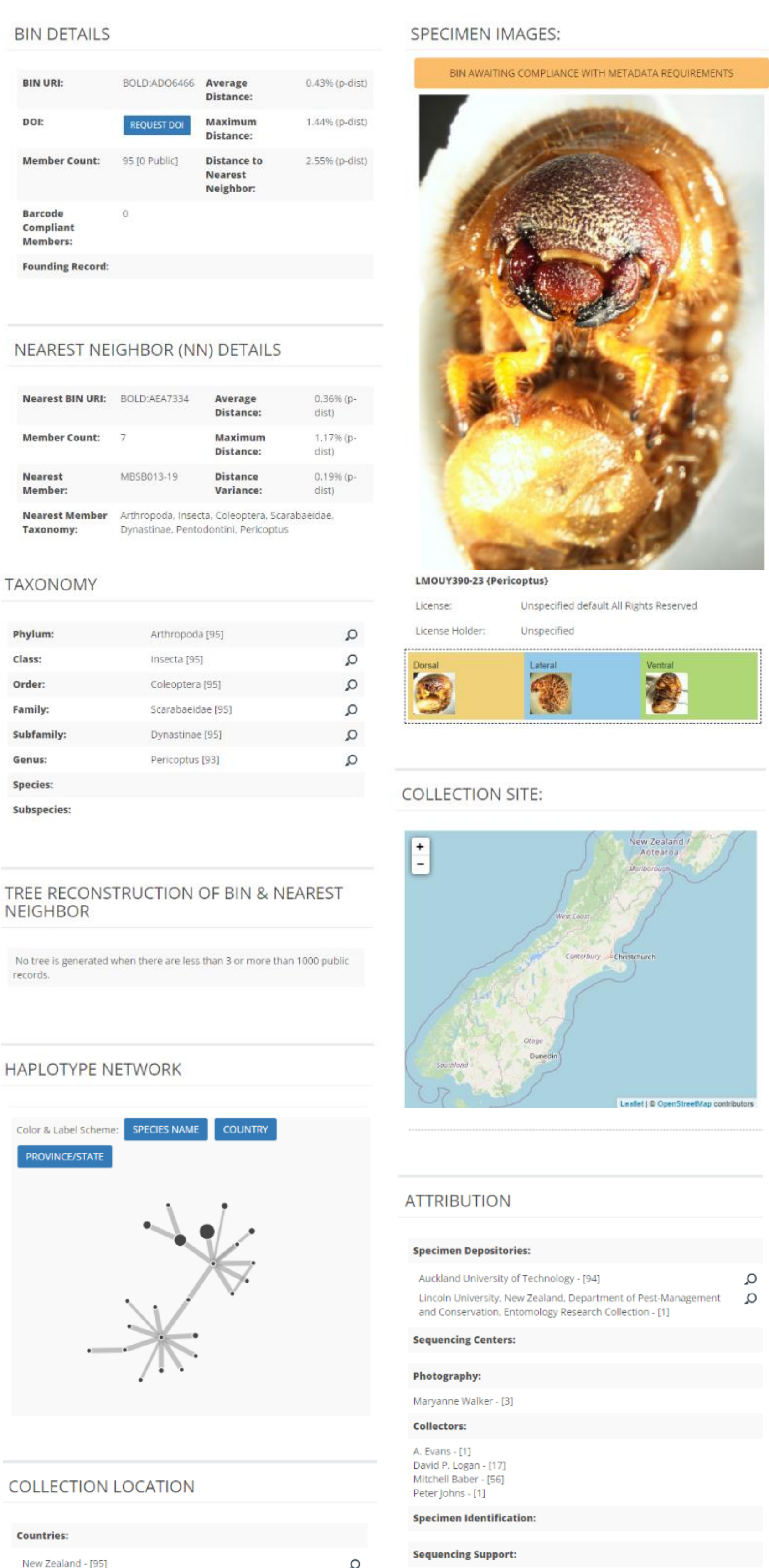


Figure 4-5. Example BIN page from BOLD systems for sample NZ750_2023 - *Pericoptus* sp.

4.5 Discussion

I sequenced 50 invertebrate specimens from Kaitorete Spit, of which, 41 were successful. The success of a sequence is determined by sample quality, contamination during the PCR process, primer binding, and the presence of an inhibitory compound (Lear et al. 2017). The efficient use of databases is reliant on the information in them (Collins et al. 2021). One match in my study, the southern ant *Chelaner antarcticus* (Forel, 1892), was identified in GenBank as an inactive taxon *Monomorium antarcticum.* The DNA record is stored at *M. antarcticum* but the name has since been changed to *C. antarcticus* (Lancaster 2022). If these taxonomic changes are not detected in larger samples, it can cause misidentification or incorrect information about species distributions.

Each successfully sequenced specimen was graded on how well it matched the identification available on GenBank. Of the species with a high grade >99%, seven were from introduced species, and the others are the common New Zealand grass moth species (Family: Cambidae) and the New Zealand garden wolf spider (*Anoteropsis hilaris*) that have been sequenced as part of previous studies. The *A. hilaris* sequence was published as part of a study to determine the phylogeny and arrival of the species in New Zealand (Vink & Paterson 2003). The grass moth species were submitted as part of a direct submission to GenBank for voucher specimens (GenBank: *Eudonia submarginalis* LR135682.1, *Achyra affinitalis* HQ952520.1). This shows the importance of uploading sequences to assist future studies looking to use barcodes as it allows confirmation of sequences or decreases the number of new sequences needed.

The remaining 14 sequences were graded between 97% and 80% for their family, genus or species identification, indicating that the correct identification may not be available on GenBank (Verkuil et al. 2022) and that these are new sequences to the database. The poor matches highlight the need for more cataloguing of species in New Zealand. Creating this library of invertebrate sequences will prevent misidentification in Chapter 5, which can be caused by gaps in reference libraries. An analysis of aquatic macroinvertebrates in Apulia, Italy highlights the issue when databases are incomplete and create gaps in global databases, such as BOLD systems and GenBank (Specchia et al. 2020). It was determined that 42.3% of the 1546 examined specimens lacked DNA barcodes, and only 52% of specimens with barcodes had reported primers (Specchia et al. 2020). Incomplete records reduce the effective use of DNA databases in future studies.

An example of a complete reference library leading to success in a dietary study is shown by research on the diet of the New Zealand native land snail (*Powelliphanta augusta)* (Boyer et al. 2013). Sequences from 139 earthworm specimens, representing 16 species, were sequenced from the known habitat of the snail to maximise the taxonomic representation of earthworm species (Boyer et

al. 2013). The snail's diet included 14 of the 16 species, showing that the snails have a broad range of earthworms in their diet (Boyer et al. 2013). This diversity in snail diet will influence where the snails are translocated to during restoration projects and what to feed captive snails to represent wild diets. This information would not have been possible without the creation of the DNA library of the dietary earthworms.

DNA barcodes can also assist in efficient biosecurity monitoring and diagnosis of invasive species. deWaard et al. (2010) demonstrated the importance of DNA barcodes for identifying species of *Lymantria* spongy moth, which can be an important forestry pest in radiata pine plantations (Castedo-Dorado et al. 2016). The study found a success of 97.2% (one failure) in distinguishing between 36 species using the COI gene, concluding further library expansion will increase its use in the diagnosis and monitoring of taxa of concern (deWaard et al. 2010).

4.6 Conclusion

The formation of this DNA library successfully created 41 invertebrate barcodes for use in New Zealand-specific invertebrate studies, and specifically for our survey of Kaitorete Spit. Reference libraries are important in providing site-specific DNA sequences that otherwise would not be represented in public databases. This DNA library will be used, along with other existing sequences, in Chapter 5 of my thesis to compare the output from a small, local collection and global databases.

References

Bell, M. (2017). Population size, breeding success and predators of black-fronted tern (*chlidonias albostriatus*) in the Upper Clarence River catchment, New Zealand. *Notornis, 64*(3), 154–161.

Bogich, T. L., Barker, G. M., Mahlfeld, K., Climo, F., Green, R., & Balmford, A. (2012). Fragmentation, grazing and the species–area relationship. *Ecography, 35*(3), 224–231. https://doi.org/https://doi.org/10.1111/j.1600-0587.2011.07136.x

Boyer, S., Wratten, S. D., Holyoake, A., Jawad, A., & Cruickshank, R. H. (2013). Using Next-Generation Sequencing to analyse the diet of a highly endangered land snail (*Powelliphanta augusta*) Feeding on Endemic Earthworms. *PLOS ONE, 8*(9). https://doi.org/https://doi.org/10.1371/journal.pone.0075962

Cardinale, B. J., Duffy, J. E., Gonzalez, A., Hooper, D. U., Perrings, C., Venail, P., Narwani, A., Mace, G. M., Tilman, D., Wardle, D. A., Kinzig, A. P., Daily, G. C., Loreau, M., Grace, J. B., Larigauderie, A., Srivastava, D. S., & Naeem, S. (2012). Biodiversity loss and its impact on humanity. *Nature, 486*(7401), 59–67. https://doi.org/https://doi.org/10.1038/nature11148

Castedo-Dorado, F., Lago-Parra, G., Lombardero, M. J., Liebhold, A. M., & Álvarez-Taboada, M. F. (2016). European gypsy moth (*Lymantria dispar dispar* L.) completes development and defoliates exotic radiata pine plantations in Spain. *New Zealand Journal of Forestry Science, 46*(1), 18. https://doi.org/10.1186/s40490-016-0074-y

Christopher, C. C., & Cameron, G. N. (2012). Effects of invasive amur honeysuckle (*Lonicera maackii*) and white-tailed deer (*Odocoileus virginianus*) on litter-dwelling arthropod communities. *American Midland Naturalist, 167*(2), 256–272. https://doi.org/10.1674/0003-0031-167.2.256

Collins, R. A., Trauzzi, G., Maltby, K. M., Gibson, T. I., Ratcliffe, F. C., Hallam, J., Rainbird, S., Maclaine, J., Henderson, P. A., Sims, D. W., Mariani, S., & Genner, M. J. (2021). Meta-Fish-Lib: A generalised, dynamic DNA reference library pipeline for metabarcoding of fishes. *Journal of Fish Biology, 99*(4), 1446–1454. https://doi.org/https://doi.org/10.1111/jfb.14852

Deagle, B. E., Thomas, A. C., McInnes, J. C., Clarke, L. J., Vesterinen, E. J., Clare, E. L., Kartzinel, T. R., & Eveson, J. P. (2019). Counting with DNA in metabarcoding studies: How should we convert sequence reads to dietary data? *Molecular Ecology, 28*(2), 391–406. https://doi.org/https://doi.org/10.1111/mec.14734

deWaard, J. R., Levesque-Beaudin, V., deWaard, S. L., Ivanova, N. V., McKeown, J. T. A., Miskie, R., Naik, S., Perez, K. H. J., Ratnasingham, S., Sobel, C. N., Sones, J. E., Steinke, C., Telfer, A. C., Young, A. D., Young, M. R., Zakharov, E. V., & Hebert, P. D. N. (2019). Expedited assessment of terrestrial arthropod diversity by coupling Malaise traps with DNA barcoding. *Genome, 62*(3), 85–95. https://doi.org/10.1139/gen-2018-0093

deWaard, J. R., Mitchell, A., Keena, M. A., Gopurenko, D., Boykin, L. M., Armstrong, K. F., Pogue, M. G., Lima, J., Floyd, R., Hanner, R. H., & Humble, L. M. (2010). Towards a global barcode library for Lymantria (Lepidoptera: Lymantriinae) tussock moths of biosecurity concern. *PLOS ONE, 5*(12), e14280. https://doi.org/10.1371/journal.pone.0014280

Donovan, B. J. (2007). *Fauna of New Zealand 57: Apoidea (Insecta: Hymenoptera)*. Manaaki Whenua Press.

Emami-Khoyi, A., Hartley, D. A., Paterson, A. M., Boren, L. J., Cruickshank, R. H., Ross, J. G., Murphy, E. C., & Else, T.-A. (2016). Identifying prey items from New Zealand fur seal (*Arctocephalus forsteri*) faeces using massive parallel sequencing. *Conservation Genetics Resources, 8*(3), 343–352. https://doi.org/10.1007/s12686-016-0560-9

Gaskin, D. E. (1975). Revision of the New Zealand Crambini (Lepidoptera: Pyralidae: Crambinae). *New Zealand Journal of Zoology, 2*(3), 265–363. https://doi.org/10.1080/03014223.1975.95178

Gonçalves, J., Marks, C. A., Obendorf, D., Amorim, A., & Pereira, F. (2014). The risks of using "species-specific" PCR assays in wildlife research: The case of red fox (*Vulpes vulpes*) identification in Tasmania. *Forensic Science International: Genetics, 11*, 9–11. https://doi.org/https://doi.org/10.1016/j.fsigen.2014.03.009

Hebert, P. D. N., Braukmann, T. W. A., Prosser, S. W. J., Ratnasingham, S., deWaard, J. R., Ivanova, N. V., Janzen, D. H., Hallwachs, W., Naik, S., Sones, J. E., & Zakharov, E. V. (2018). A sequel to Sanger: amplicon sequencing that scales. *BMC Genomics, 19*(1), 219. https://doi.org/10.1186/s12864-018-4611-3

Hebert, P. D. N., Floyd, R., Jafarpour, S., & Prosser, S. W. J. (2023). Barcode 100K specimens: in a single nanopore run. *bioRxiv*. https://doi.org/10.1101/2023.11.29.569282

Hetherington, J., & Wilson, J. B. (2014). Spatial associations between invasive tree lupin and populations of two katipo spiders at Kaitorete Spit, New Zealand. *New Zealand Journal of Ecology, 38*(2), 1–9. https://newzealandecology.org/nzje/3133

iNaturalist NZ. (n.d.). *Observations in New Zealand*. https://inaturalist.nz/observations

Klimaszewski, J., & Watt, J. C. (1997). *Fauna of New Zealand 37: Coleoptera*. Manaaki Whenua Press.

Lancaster, E. (2022). *Chemical and reproductive aspects of the population ecology of native new zealand ant* Monomorium antarcticum *(Fr. Smith) (Hymenoptera: Formicidae)* (Thesis, Master of Science) University of Otago. http://hdl.handle.net/10523/13478

Larivière, M.-C., Fletcher, M. J., & Larochelle, A. (2010). *Fauna of New Zealand 63: Auchenorrhyncha (Insecta: Hemiptera)*. Manaaki Whenua Press.

Larivière, M. C. (1995). *Fauna of New Zealand 35: Cydnidae, Acanthosomatidae, and Pentatomidae (Insecta: Heteroptera)* Manaaki Whenua Press.

Larochelle, A., & Larivière, M. C. (2007). *Fauna of New Zealand 60: Carabidae (Insecta: Coleoptera)*. Manaaki Whenua Press.

Lear, G., Dickie, I., Banks, J., Boyer, S., Buckley, H., Buckley, T., Cruickshank, R., Dopheide, A., Handley, K., Hermans, S., Kamke, J., Lee, C., MacDiarmid, R., Morales, S., Orlovich, D., Smissen, R., Wood, J., & Holdaway, R. (2017). Methods for the extraction, storage, amplification and sequencing of DNA from environmental samples. *New Zealand Journal of Ecology*. https://doi.org/10.20417/nzjecol.42.9

Mora, C., Tittensor, D. P., Adl, S., Simpson, G. B., & Worm, B. (2011). How many species are there on Earth and in the ocean. *PLoS Biology, 9*(8). https://doi.org/10.1371/journal.pbio.1001127

Paquin, P., Vink, C. J., & Dupérré, N. (2010). *Spiders of New Zealand: annotated family key & species list*. Lincoln, N.Z: Manaaki Whenua Press.

Ratcliffe, B. C., & Orozco, J. (2009). A review of the biology of *Pericoptus truncatus* (Fabr.) (Coleoptera: Scarabaeidae: Pentodontini) from New Zealand and a revised description of the third instar. *The Coleopterists Bulletin, 63*(4), 445–451. http://www.jstor.org.ezproxy.lincoln.ac.nz/stable/40389467

Saunders, M. E., Janes, J. K., & O'Hanlon, J. C. (2019). Moving on from the insect apocalypse narrative: Engaging with evidence-based insect conservation. *BioScience, 70*(1), 80–89. https://doi.org/10.1093/biosci/biz143

Specchia, V., Tzafesta, E., Marini, G., Scarcella, S., Simona, D. A., & Pinna, M. (2020). gap analysis for DNA barcode reference libraries for aquatic macroinvertebrate species in the Apulia Region (Southeast of Italy). *Journal of Marine Science and Engineering, 8*(7), 538. https://doi.org/https://doi.org/10.3390/jmse8070538

Stork, N. E. (2018). How many species of insects and other terrestrial arthropods are there on Earth? *Annual review of entomology, 63*(1), 31–45. https://doi.org/10.1146/annurev-ento-020117-043348

Stork, N. E., McBroom, J., Gely, C., & Hamilton, A. J. (2015). New approaches narrow global species estimates for beetles, insects, and terrestrial arthropods. *Proceedings of the National Academy of Sciences, 112*(24), 7519–7523. https://doi.org/10.1073/pnas.1502408112

Thomsen, P. F., & Willerslev, E. (2015). Environmental DNA – An emerging tool in conservation for monitoring past and present biodiversity. *Biological Conservation, 183*, 4–18. https://doi.org/https://doi.org/10.1016/j.biocon.2014.11.019

Verkuil, Y. I., Nicolaus, M., Ubels, R., Dietz, M. W., Samplonius, J. M., Galema, A., Kiekebos, K., de Knijff, P., & Both, C. (2022). DNA metabarcoding quantifies the relative biomass of arthropod taxa in songbird diets: Validation with camera-recorded diets. *Ecology and Evolution, 12*(5), e8881. https://doi.org/https://doi.org/10.1002/ece3.8881

Vink, C. J. (2002). *Fauna of New Zealand 44: Lycosidae (Arachnida: Araneae)*. Manaaki Whenua Press.

Vink, C. J., & Paterson, A. M. (2003, Sep). Combined molecular and morphological phylogenetic analyses of the New Zealand wolf spider genus Anoteropsis (Araneae: Lycosidae). *Mol Phylogenet Evol, 28*(3), 576–587. https://doi.org/10.1016/s1055-7903(03)00219-7

Walker, A. K., & Crosby, T. K. (1988). *The Preparation and Curation of Insects*. Science Information Publishing Centre, DSIR. https://books.google.co.nz/books?id=SXqpyQEACAAJ

Ward, D. (2024). *Key to the ants of New Zealand*. Manaako Whenua Landcare Research. https://www.landcareresearch.co.nz/discover-our-research/biodiversity-biosecurity/plants-invertebrates-fungi-and-bacteria/invertebrate-systematics/ants-wasps-and-bees/key-to-the-ants-of-nz/

Watt, J. C. (1992). *Fauna of New Zealand 26: Tenebrionidae (Insecta: Coleoptera)*. Manaaki Whenua Press.

Wiens, J. J. (2023). How many species are there on Earth? Progress and problems. *PLoS Biol, 21*(11). https://doi.org/10.1371/journal.pbio.3002388

Chapter 5

European hedgehog (*Erinaceus europaeus*) diet in New Zealand determined using high throughput sequencing of faecal DNA.

5.1 Introduction

The New Zealand ecosystem is at threat from many introduced mammalian predators due to intentional and accidental introductions. Analysing introduced animal diets can provide important information about what they feed on as a method to assess ecological damage (Ogle 1997), establish restoration projects (Didham et al. 2009), and population management plans (Keedwell et al. 2002).

The impact of introduced animals on the unique native flora and fauna in New Zealand has been shown through numerous dietary studies. For example, mammalian pests like the brushtail possum (*Trichosurus vulpecula)* change the composition of plant species in a forest through selective browsing on plant foliage, such as *Aristotelia serrata* (J.R. & G.Forst.) W.R.B.Oliv. (wineberry) and *Muehlenbeckia australis* (G.Forst.) Meisn. (pohuehue), and possums also feed on other food resources such as invertebrate larvae (Owen & Norton 1995). Mustelids (stoats (*Mustela erminea*), ferrets (*M. furo*), and weasels (*M. nivalis*)) were introduced to control rabbit populations and now predate on a wide array of native taxa in New Zealand (National Pest Control Agencies 2018) and cause significant damage to the native ecosystem. Mustelids are one of the major introduced predator threats to ground-nesting bird eggs, juveniles, and adults (Dowding & Murphy 2001; Sanders & Maloney 2002). In a study of the diet of mustelid species in New Zealand, Murphy et al. (1998) identified several species of rodents, birds, and invertebrates from stomach contents. Body parts of lizards, particularly in stoat and weasel stomachs, and lagomorph body parts have been shown in stoat and ferret stomach contents (Murphy et al. 1998). Bird, lizard, and invertebrate body remnants have been found in stoat faeces (38%, 10% and 50% respective occurrence frequency) and stoat dens (70%, >1%, and >5% respective occurrence frequency) (Dowding et al. 2015). Miller and Miller (1995) showed similar results in a study of the diet of rodents (i.e., ship rats (*Rattus rattus*) and mice (*Mus musculus*)) where stomach contents mainly consisted of invertebrates throughout the year, with plant matter contributing to a minor part of the diet. Rats have also been reported to prey on endemic frogs (*Leiopelma* spp. Fitzinger, 1861) (Egeter et al. 2019).

Hedgehogs (*Erinaceus europaeus* Linnaeus, 1758) were introduced into New Zealand to control garden pests in the 1870s (Bull 1968). Consequently, hedgehogs escaped and established into other habitats, both urban and native, and readily fed on a wider range of native invertebrates across New Zealand (Brockie 1959; Campbell 1973; Foster et al. 2021). Morphological analysis has shown

hedgehogs mainly feed on invertebrates (Nottingham et al. 2019) including a high proportion of beetles (present in 81% of 192 stomach samples) (Jones et al. 2005). Hedgehogs are also known to feed on bird eggs, chicks and lizards in smaller abundances (Jones et al. 2005). Despite knowing the predation habits of hedgehogs, and an increase in the recognition that hedgehogs are potentially a serious threat to New Zealand biodiversity, there are no standardised best-practice control methods to manage introduced hedgehogs (Jones 2019). Historically, hedgehogs were considered of secondary importance and reported as incidental captures (by-catch) during the control of other mammalian predators (Jones 2006). In recent research on hedgehog control, Jones et al. (2021) found that some traps commonly used (DOC 200 and 150) traps did not allow entry of average and large sized hedgehogs. Effectively targeting only small hedgehogs could have serious implications for management plans where the larger hedgehogs, which are more likely to survive winter, are not controlled (Jones et al. 2021).

Earlier studies on the diet of hedgehogs in New Zealand were based on visual identification of diagnostic stomach contents in Auckland (Nottingham et al. 2019), the Mackenzie Basin (Fountain et al. 2013; Jones et al. 2005; Moss 1999), Hawkes Bay (Jones 2006), and Northland (Hendra 1999). Similarly, visual analysis of hedgehog faecal content has been reported from along the Clutha River (Jones & Norbury 2011) and in Hawkes Bay, in addition to a small sample of stomachs (Berry 1999). While the visual identification of stomach contents provides useful information for a species diet, these methods usually require expertise in both morphology and microscopy. Additionally, species without recognisable hard body parts are often overlooked, resulting in a biased estimation of diet (Monterroso et al. 2019).

Recent developments in DNA sequencing technology and computing platforms have made it possible to obtain comparable dietary information from stomach and faecal contents that remain in the environment (eDNA) (Ficetola et al. 2010; Thomsen & Willerslev 2015). The molecular analysis of a species diet also allows for better identification of soft-bodied dietary items that are often overlooked in most morphological analysis (Emami-Khoyi et al. 2016; Jones 2006; Monterroso et al. 2019). The detectability of dietary items has been shown to significantly increase when using molecular methods compared to traditional morphological methods for detecting the southern bell frog (*Litoria raniformis* (Keferstein, 1867)) in mammalian faeces (0% to 50%) and stomachs (2% to 70%) (Egeter et al. 2015). Molecular techniques have also shown to be successful in the identification of dietary items, even after long digestion times, when it is not possible to visually identify hard parts remnants due the fragmentation of contents during digestion (Carreon-Martinez et al. 2011). To successfully detect dietary items in stomach and faecal content, analysis methods need to target a unique, short fragment of DNA that survives digestion and is distinguishable between similar dietary items (Emami-Khoyi 2024).

The mitochondrial cytochrome oxidase c subunit I (COI) gene was selected as a DNA marker for my study because it is known to be a reliable marker in many molecular reconstructions of diets for distinguishing closely related species (Emami-Khoyi et al. 2016; Rodrigues et al. 2017; Serite et al. 2022). Some fragments of this marker are relatively short and survive digestion and remain intact (Serite et al. 2022). Previous DNA studies have determined the diet of several species, including but not limited to, pastural spiders (Vink & Kean 2013), the New Zealand fur seal (*Arctocephalus forsteri* (Lesson, 1828)) (Emami-Khoyi et al. 2016), pipefish (Serite et al. 2022), and herbivorous invertebrates (Matheson et al. 2008).

In this study, I targeted DNA from the stomach and faecal content of the European hedgehog to reconstruct the hedgehog diet. The chosen method of molecular analysis (High Throughput Sequencing (HTS)) has not been used to reconstruct the entire diet of hedgehogs in New Zealand before. Other molecular methods have been used with DNA from hedgehog stomach and faecal contents to target a specific species and have been successful (Egeter et al. 2015). This chapter aims to evaluate the performance of HTS methods for the stomach and faecal contents of hedgehog and investigate if the presence of some taxa in the diet may have been unintentionally overlooked using traditional hard part analysis. This thesis will build on the existing evidence of the negative impact of hedgehogs on the local ecosystem and aim to justify the establishment more targeted management plans and standardise many of the current control protocols.

5.2 Objectives

5.2.1 Undertake metabarcoding analysis of hedgehog faecal and colon content to determine the diet of hedgehogs on Kaitorete Spit.

5.2.2 Evaluate the taxonomic assignment of sequences from the locally generated reference database of sequences (see Chapter 4) to sequences generated through metabarcoding to identify site-specific invertebrates that may not have had publicly databased sequences available.

5.2.3 Analyse the potential for this methodology to be used as a complementary monitoring method to be integrated into the current predator control plans on Kaitorete Spit.

5.3 Methods

5.3.1 Faecal sample collection

Hedgehog faeces were collected from Kaitorete Spit (43.82°S, 172.60°E) during May 2023. Searches for samples were made by foot along fence lines of grazed paddocks and vehicle tracks covered with short grass. Discovered faeces were photographed, put into a sterile vial, and labelled with date and location, then frozen in a -20 °C freezer on return from the field (<5 hours). A subset of five of the heaviest faeces (1.5–2.8 g) were selected for further analysis and two spare faeces (>1.1 g) were also collected.

5.3.2 Colon collection

Hedgehog colon samples were collected from 10 hedgehogs as outlined in Chapter 3. A subset of the five heaviest colon contents (2.8–4.6 g) were selected along with two spare colon contents (>1.7 g). One sample weighed >10 g, which exceeded the volume of the DNA extraction kit, and a subsample of <10 g was taken after homogenising the sample by mixing it together with a sterile pipette tip (outlined in Chapter 3.3.2).

5.3.3 DNA extraction

DNA from five hedgehog faecal and colon contents were extracted using a QIAGEN DNeasy Power Max soil Kit, as outlined in Chapter 2. The extracted DNA product was run on a 0.5% agarose gel for 15–30 minutes at 100 volts then visualised on a Bio Rad GelDoc Go Gel Imaging System.. A volume of 2 mL of extracted DNA was sent to the Austrian Centre of Technology (AIT) for DNA analysis.

5.3.4 High Throughput Sequencing (HTS)

Meta-barcode sequencing was performed at AIT following the protocol outlined in Serite et al. (2022) and is summarised below. The AIT laboratory used for library preparation complies with the requirements of standard ISO 9001:2015 and workbenches and equipment were cleaned with bleach before analysis. The PCR setup was undertaken in a physically separate laboratory within a HEPA-filtered laminar flow chamber and no-template controls (NTCs) were incorporated into the same workflow to monitor potential contamination. The mitochondrial cytochrome oxidase c subunit I (COI) gene was amplified using the forward primer mlCOIintF and reverse primer jgHCO2198 (Leray et al. 2013) as described in (Ntuli et al. 2020). The COI gene is typically diverse enough to differentiate between animal species and its performance has been successfully tested for DNA from various animal species (Hebert et al. 2003) including several invertebrate species (Elbrecht et al. 2019) that are known to be important taxa in the hedgehog diet (e.g. Coleoptera) (Yalden 1976). The primer set used in this study has a higher success rate for sequencing DNA than other versatile

primers designed for the COI gene that target longer fragments of DNA that would not survive digestion e.g., LCO1490/HCO2198 (Leray et al. 2013). Elbrecht et al. (2019) recommended targeting short fragments of 200 bp for degraded DNA and King et al. (2008) recommended fragments <300 bp for DNA extraction from faecal and stomach samples.

The PCR thermal protocols were similar to those reported by Serite et al. (2022). Prior to the high throughput sequencing, PCR products were purified using the AMPure XP system (Beckman Coulter), and a NEBNext Ultra DNA Library Prep Kit (New England BioLabs, United States) was used for the preparation of metagenomic libraries. The resulting libraries were then sequenced on an Illumina MiSeq platform ((Illumina Inc., San Diego, California, United States) using 2x300 bp paired-end chemistry according to the manufacturer's instructions.

5.3.5 Bioinfortmatics Analysis

Quality Control

The quality of the raw DNA sequences was visually inspected using FastQC v.012 (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and summarised using MultiQC v1.20 (Ewels et al. 2016). In the metabarcoding analysis, three mandatory steps were required to assemble the amplicons before assigning taxonomic rank. In the first step, low-quality sequences (i.e., those with a Phred score below a user-defined value, typically between 20–30), sequencing primers, and adaptors were filtered out. Next, quality filter sequences were merged based on the overlap between forward and reverse sequences. These merged sequences are de-noised and de-replicated into the number of unique sequences, and the abundance for each identical sequence clusters were recorded. Finally, chimeric sequences that are PCR artefacts rather than sequences of biological origin were removed and the remaining sequences were clustered based on a user-defined similarity threshold, typically 97–98%, since sequences from identical genera are expected to have a similarity within that range. While these steps are usually common among all analytical pipelines for metabarcoding, different variants that slightly differ in order and naming protocols exist. In my study, I use the Anacapa Toolkit (Curd et al. 2019), which is a collection of widely used packages, to analyse environmental DNA from quality filtering to taxonomic rank assignments.

Briefly explaining the Anacapa Toolkit requires a specifically formatted and taxonomically curated reference database that allows the users to match the generated sequences to a specific taxonomic level. In the Anacapa Toolkit, the CRUX (Creating Reference libraries Using eXisting tools) pipeline (Curd et al. 2019) generates a reference database for any user-defined markers by first performing a series of *in silico* PCRs (echo PCRs (Ficetola et al. 2010)) on the EMBL (European Molecular Biology Laboratory) nucleotide databases to identify a series of seeds each with unique identifiers. These

seed libraries are then searched against the larger NCBI nucleotide BLAST dataset (https://blast.ncbi.nlm.nih.gov/Blast.cgi) in two consecutive steps. In the first step, the BLAST only reports the full-length matches. In the second step, BLAST accepts reads with matches of up to 70%. During CRUX database generation steps, the presence of the correct primer regions was assessed, and these primers were subsequently trimmed using Cutadapt (Martin 2011). In parallel, a taxonomy identification file corresponding to the known taxonomic rank (e.g., superkingdom, phylum, class, order, family, genus, species) was generated using entrez_qiime (https://github.com/bakerccm/entrez_qiime). However, since many publicly available records on NCBI are not complete, and might include taxonomic ranks such as uncultured, unknown, unassigned or "NA", the Anacapa pipeline created two databases in parallel, filtered, and unfiltered. I expected that sequences from some of the major taxa found in the study area would not be publicly available and had not been sequenced previously. To overcome the absence of some sequences, I created a local barcoding database from the taxa that I could trap and subsequently identify in the area (see Chapter 4). Without this local database, the lack of local sequences could negatively affect taxonomic rank assignment by reporting prey item taxa that are not found in New Zealand as the best matches. These in-house generated sequences were amended (https://github.com/limey-bean/CRUX_Creating-Reference-libraries-Using-eXisting-tools) to a COI reference library generated in October 2023 from all publicly available records and this combined reference dataset was used throughout this chapter for taxonomic tank assignment.

Construction of Amplicon Sequence Variation

Amplicon sequence variation (ASV) is a new addition to a wide array of methodologies to identify similar biological sequences. The statistical method used for ASV assignment is different from the concept of Operational Taxonomic Units (OTU), which has been widely used in metabarcoding studies. While the technical detail of this difference is beyond the scope of this thesis, briefly, the major difference between OTUs and ASVs is that in traditional OTU assignments, sequences are clustered based on arbitrary user-defined sequence similarity thresholds (Jeske 2022).However, the underpinning statistical framework to assign ASV is based on complex Bayesian methods that take into consideration the observed level of sequencing error in a database to collapse identical sequences. As such, the developer of these methods suggests that sequences that are as little as two base pairs apart can be reliably identified, and this results in a more accurate taxonomic rank assignment and more precise estimation of the observed diversity within ecological communities (Callahan et al. 2016).

The Anacapa pipeline used the DADA2 software package (Divisive Amplicon Denoising Algorithm) to process raw sequences. Before this step, sequencing adaptors and low-quality sequences were identified and removed using a combination of Cutadapt (Martin 2011) and FastX Toolkit (Gordon &

Hannon 2010). Only the subset of sequences that had a Phred score >35 probability (with an error rate <0.001) and a minimum length of 100 bp were selected for DADA2 analysis. A Python script passed quality filtered sequences into DADA2 where the unpaired forward, unpaired reverse, and unmerged sequences were processed separately. DADA2 de-noises, de-replicates, and, when possible, merges forward and reverse sequences. Similarly, chimeric sequences were identified and subsequently removed from analysis.

Taxonomic rank assignment

The final step of the Anacapa Toolkit is to assign a taxonomic rank to the constructed ASVs. For this purpose, Anacapa uses a pipeline that is based on a combination of read mapping using Bowtie 2 short read aligner (Langmead & Salzberg 2012) and Bayesian-based last common ancestor (BLCA) taxonomic classification methods implemented in the BLCA package (Gao et al. 2017). BLCA is unique in terms of providing a solid probabilistic method for taxonomic rank assignment to query sequences.

Result visualisation and diversity estimation

The assigned taxonomic ranks, basic alpha and beta diversity indices, and the Jaccard indices of dissimilarity were visualised using a combination of rAnacapa (https://github.com/gauravsk/ranacapa) and phylotool v.2.0 tools R package (https://github.com/helixcn/phylotools (Revell 2024)). Observed and Shannon diversity was used to analyse the alpha diversity to account for both the number of species and relative abundance of each group. For the beta diversity, the Jaccard index was used over Bray-Curtis index due eDNA-based abundance data not always being reliable (https://gauravsk.shinyapps.io/ranacapa/).

5.4 Results

5.4.1 Raw DNA visualization and data.

The raw DNA extracted from samples were consistent and there was no observation of major degradation that makes DNA unusable for amplification and sequencing. An example gel electrophoresis of two faeces and five colons is shown in Figure 5-1. The Nanodrop results also confirmed that DNA of desirable quality was extracted from hedgehog faecal and colon sources (Table 5-1).

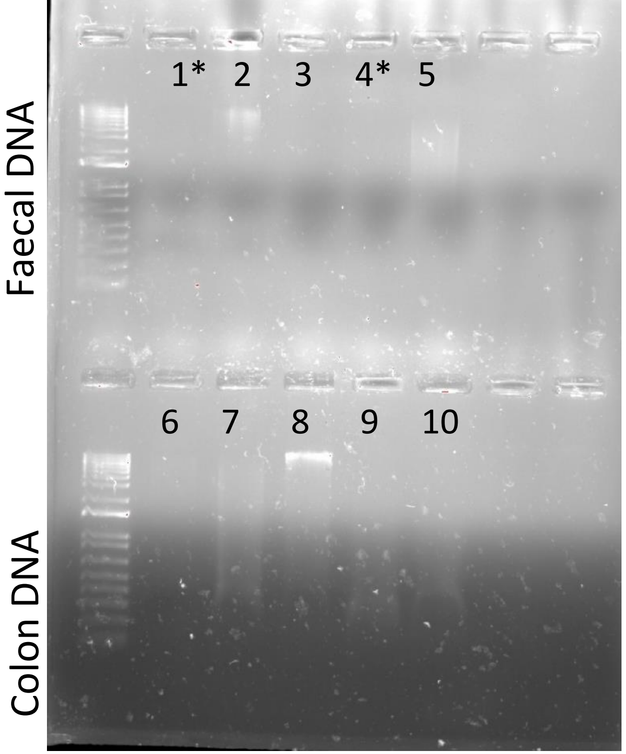


Figure 5-1. Gel electrophoresis from crude DNA was extracted and sent to Austria for high-throughput sequencing analysis. *Indicates samples that did not amplify in PCR.

Table 5-1. Nanodrop readings of quality, quantity, and raw sample weight.

Code	Quantity ng/nL	Quality A260/A280	Weight g
1 Faeces	19.9	1.39	1.56
2 Faeces	22.7	1.71	1.67
3 Faeces	5.5	1.30	1.92
4 Faeces	6.3	1.39	1.98
5 Faeces	28.0	1.72	2.78
6 Faeces	NA	NA	1.15
7 Faeces	NA	NA	1.43
6 Colon	14.3	1.34	4.63
7 Colon	25.0	1.69	2.87
8 Colon	26.9	1.53	3.65
9 Colon	15.9	1.63	3.50
10 Colon	34.3	1.36	4.33

5.4.2 High Throughput Sequencing

The Illumina MiSeq sequencing run yielded a total of 36 million sequences with an average of 1.8 m sequences per sample. The average sequence length was 297 base pairs with an average GC content of 37% (Table 5-2). From the faecal samples analysed, only two samples failed to produce usable sequences (01.FAECES and 04.FAECES). As a result, backup samples from the same sources were amplified and successfully sequenced (06.FAECES and 07.FAECES). In total, after quality filtering, the Anacapa pipeline collapsed these sequences into 909 unique amplicon sequence variants (ASV) that were used for downstream analysis. While the Anacapa pipeline reports taxonomic rank for forward, reverse, and merged sequences separately, I decided to only report merged sequences since I considered these successfully merged sequences to be of acceptable quality to be used for the inference of results.

Table 5-2. Output from Multi QC report for each sample. R1 = forward, R2 = reverse, M Seqs = Million Sequences.

Sample Name	% Dups	% GC	Length	M Seqs
02.FAECES_R1	83.90%	32%	300	3.9
02.FAECES_R2	87.40%	32%	300	3.9
03.FAECES_R1	83.10%	34%	288	1.1
03.FAECES_R2	83.50%	34%	289	1.1
05.FAECES_R1	81.80%	34%	299	2.8
05.FAECES_R2	85.20%	34%	299	2.8
06.FAECES_R1	75.20%	34%	300	1.1
06.FAECES_R2	79.90%	34%	300	1.1
07.FAECES_R1	85.10%	36%	299	1.3
07.FAECES_R2	86.60%	36%	300	1.3
06.COLON_R1	94.60%	39%	297	2.1
06.COLON_R2	92.60%	38%	300	2.1
07.COLON_R1	93.30%	39%	297	1.8
07.COLON_R2	91.00%	39%	300	1.8
08.COLON_R1	93.10%	39%	296	1.5
08.COLON_R2	90.50%	39%	300	1.5
09.COLON_R1	89.10%	39%	296	1.2
09.COLON_R2	87.90%	39%	299	1.2
10.COLON_R1	91.90%	39%	293	1.2
10.COLON_R2	88.80%	40%	297	1.2

5.4.3 Barcoded Specimens

From the 41 in-house barcoded specimens, 12 were matched with sequences from the HTS output, representing seven species identified from the area through pitfall trapping (Table 5-3). The HTS

sequences from faecal samples matched with two locally barcoded species (Table 5-4) and the HTS sequences from the colon content matched with seven locally databased species (Table 5-5).

Table 5-3. Summary of species matches from the invertebrate library (Chapter 4) in the high throughput sequencing of colon and faecal content.

ILCode	Order	Family	Species	Common Name
NZ761	Diptera	Calliphoridae	*Calliphora vicina*	Small blowfly
NZ771	Coleoptera	Carabidae	*Mecyclothorax rufipennis*	Hawaiian beetle
NZ775	Opiliones	Phalangiidae	*Phalangium opilio*	European harvestman
NZ785	Lepidoptera	Crambidae	*Eudonia submarginalis*	NZ grass moth
NZ786	Lepidoptera	Crambidae	*Eudonia submarginalis*	
NZ787	Lepidoptera	Crambidae	*Achyra affinitalis*	Cotton web spinner
NZ788	Lepidoptera	Crambidae	*Eudonia submarginalis*	
NZ790	Lepidoptera	Crambidae	*Eudonia submarginalis*	
NZ791	Lepidoptera	Crambidae	*Achyra affinitalis*	
NZ793	Lepidoptera	Crambidae	*Orocrambus vittellus*	NZ grass moth
NZ797	Orthoptera	Trigonidiidae	*Bobilla* sp.	Bobilla
NZ799	Orthoptera	Trigonidiidae	*Bobilla* sp.	Bobilla

*NZ = New Zealand, ILCode = Invertebrate Library Code

Table 5-4. Species matches from HTS for faecal samples with the number of sequences and the percentage match to order and species identifications.

ILCode	2.FAECES	3.FAECES	5.FAECES	6.FAECES	7.FAECES	Order	%	Species	%
NZ799 NZ797	0	0	855	0	0	Orthoptera	100.00	*Bobilla*	100.00
NZ775	45	0	0	0	0	Opiliones	92.71	*Phalangium opilio*	100.00

Table 5-5. Species matches from HTS for colon content samples with the number of sequences and the percentage match to the identification.

ILCode	6.COLON	7.COLON	8.COLON	9.COLON	10.COLON	Order	%	Species	%
NZ761	0	0	1035	0	0	Diptera	98.84	*Calliphora vicina*	96.51
NZ771	0	0	0	41	0	Coleoptera	99.00	*Moriomorphini sp.*	99.00
NZ775	0	0	923	0	0	Opiliones	93.38	*Phalangium opilio*	100.00

NZ790 NZ786 NZ788 NZ785	0	0	0	0	1128	Lepidoptera	100.00	*Scoparia* sp. *Eudonia submarginalis*	100.00
NZ791 NZ787	114439	0	0	0	0	Lepidoptera	60.83	*Achyra affinitalis*	60.83
NZ793	0	0	0	164	0	Lepidoptera	100.00	*Orocrambus vittellus*	100.00
NZ799 NZ797	0	0	52	0	0	Orthoptera	100.00	*Bobilla*	100.00

5.4.4 Anacapa

Metabarcoding identified numerous taxa belonging to Arthropoda (Figure 5-3 & Figure 5-4), Annelida (Figure 5-5), Mollusca (Figure 5-6), Nematoda (Figure 5-7) and Rotifera (Figure 5-8). Among these phyla, Arthropoda with 46 genera (Figure 5-3) from 40 families (Figure 5-4), Annelida with three genera (Figure 5-5), and Nematoda with 23 genera (Figure 5-7) were the most abundant phyla, respectively. All phyla were observed from both sources but not all taxa were invariably identified from each sample. For instance, colon content had a higher abundance of Arthropoda than faecal content, while faecal content showed more diversity with higher abundances of all other phyla (Figure 5-2). Each sample is represented by an Anacapa Code (Table 5-6) in Figure 5-2 through Figure 5-14.

Table 5-6. Reference list of sample codes matching the collection code and the code after Anacapa analysis.

Collection Code	Anacapa Code
2 Faeces	Faeces.S1
3 Faeces	Faeces.S4
5 Faeces	Faeces.S3
6 Faeces	Faeces.S15
7 Faeces	Faeces.S2
6 Colon	Colon.S5
7 Colon	Colon.S7
8 Colon	Colon.S9
9 Colon	Colon.S11
10 Colon	Colon.S13

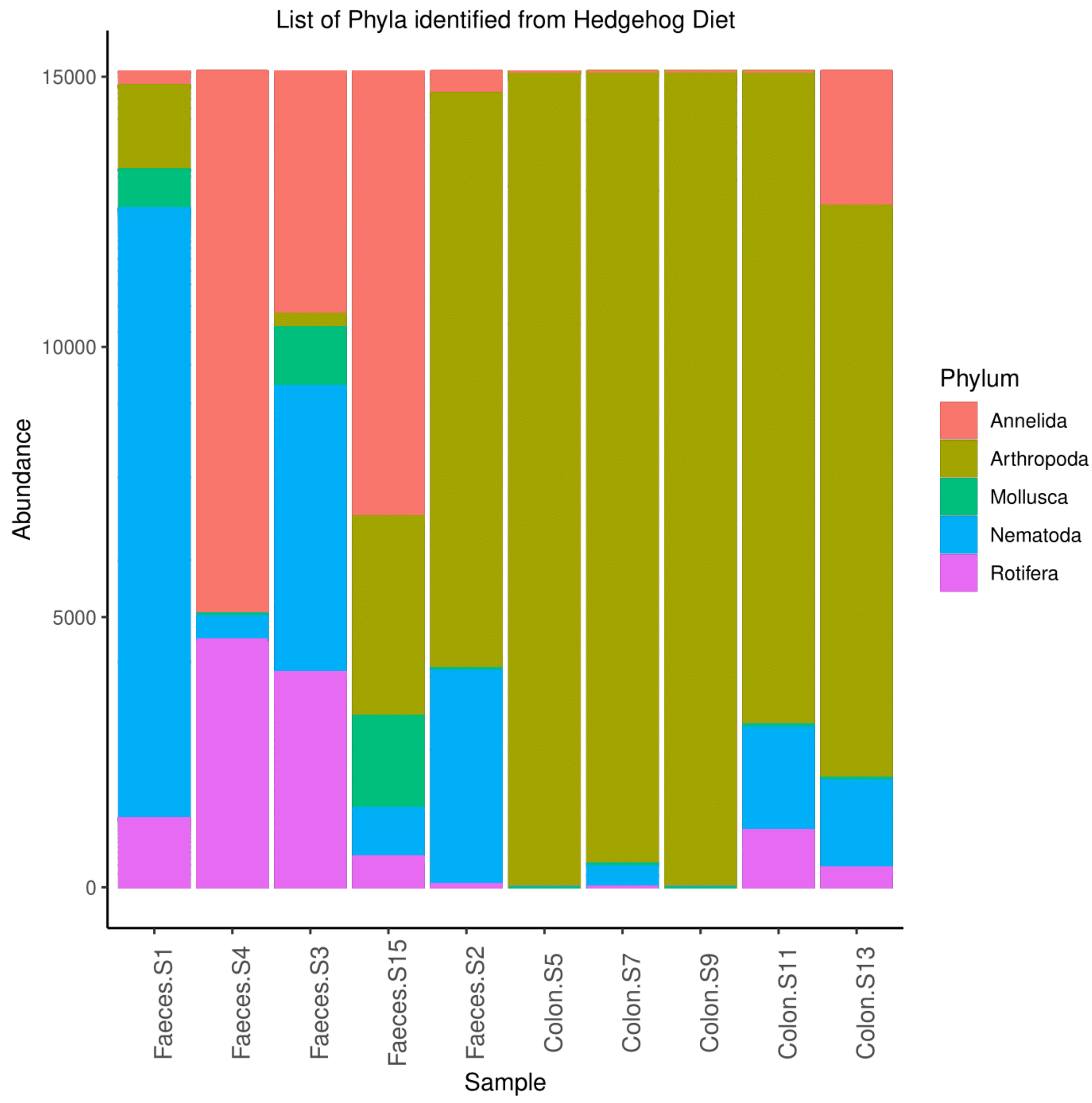


Figure 5-2. Comparison of the number of ASVs (amplicon sequence variation) at the phylum taxonomic level and the sample source (hedgehog faecal and colon content) using rAnacapa.

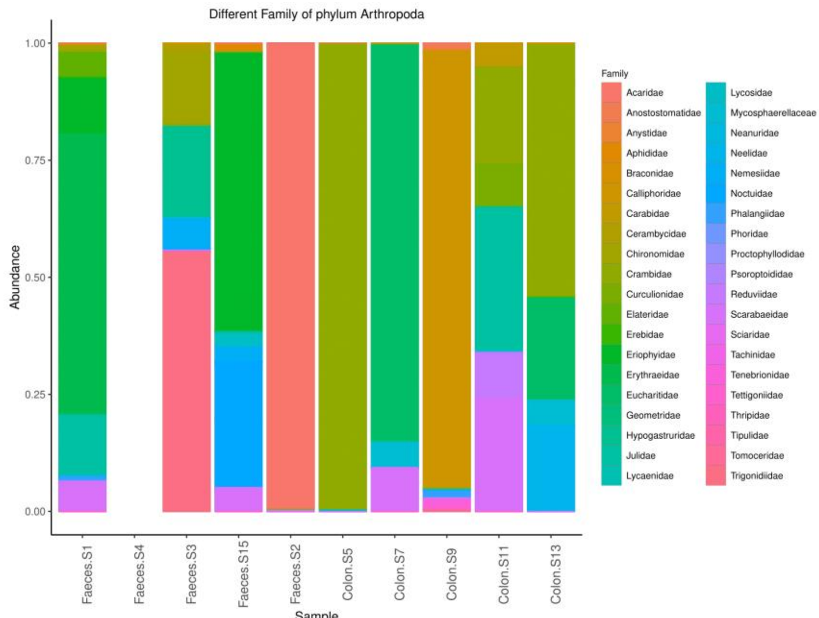


Figure 5-3. Comparison of the proportion of Arthropoda families identified through HTS (high throughput sequencing) and the sample source (hedgehog faecal and colon content) using rAnacapa.

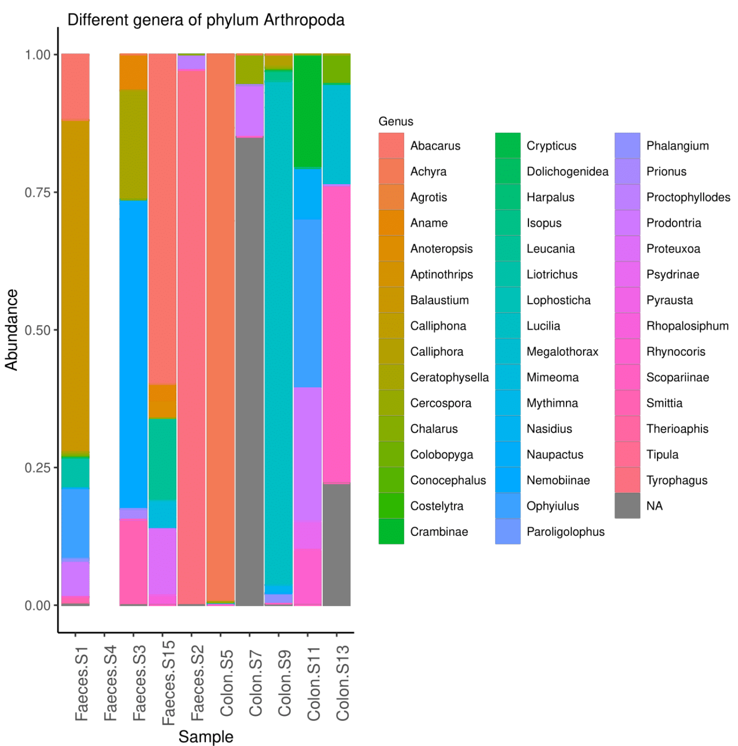


Figure 5-4. Comparison of the proportion of Arthropoda genera identified through HTS (high throughput sequencing) and the sample source (hedgehog faecal and colon content) using rAnacapa.

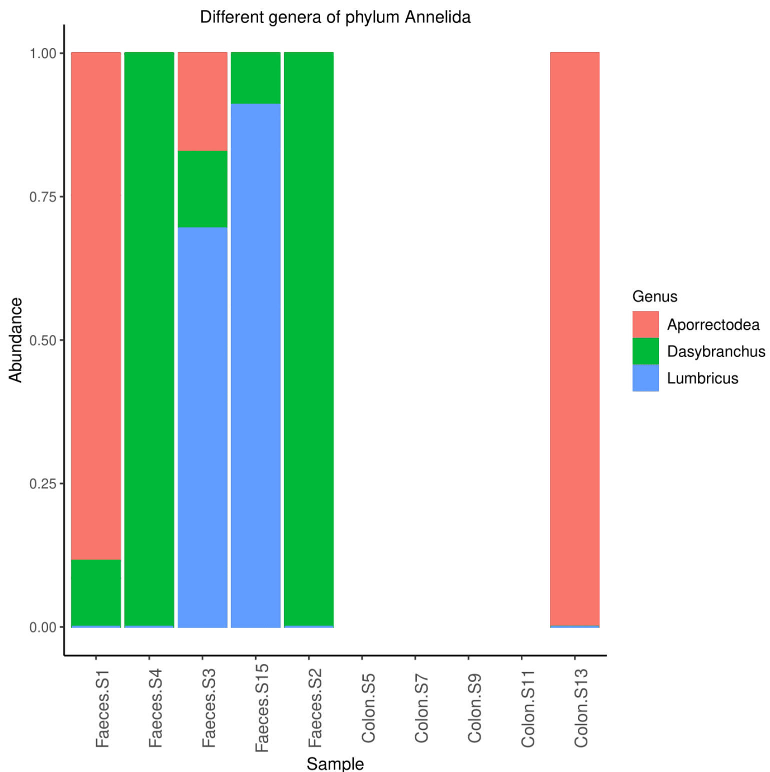


Figure 5-5. Comparison of the proportion of Annelida genera identified through HTS (high throughput sequencing) and the sample source (hedgehog faecal and colon content) using rAnacapa.

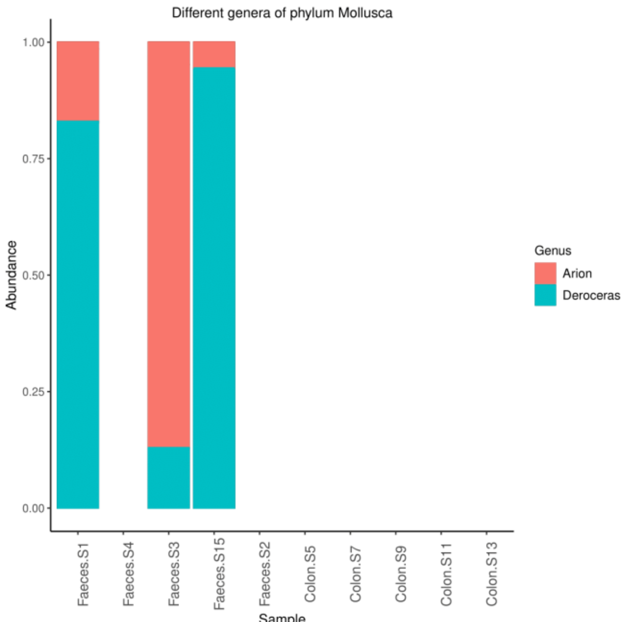


Figure 5-6. Comparison of the proportion of Mollusca genera identified through HTS (high throughput sequencing) and the sample source (hedgehog faecal and colon content) using rAnacapa.

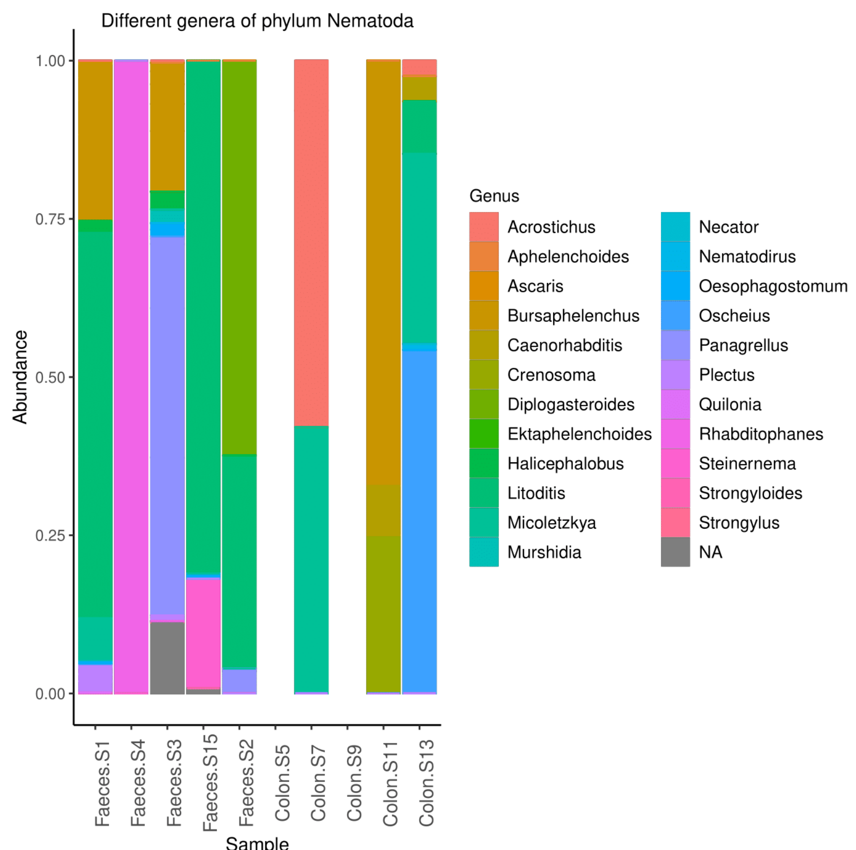


Figure 5-7. Comparison of the proportion of Nematoda genera identified through HTS (high throughput sequencing) and the sample source (hedgehog faecal and colon content) using rAnacapa.

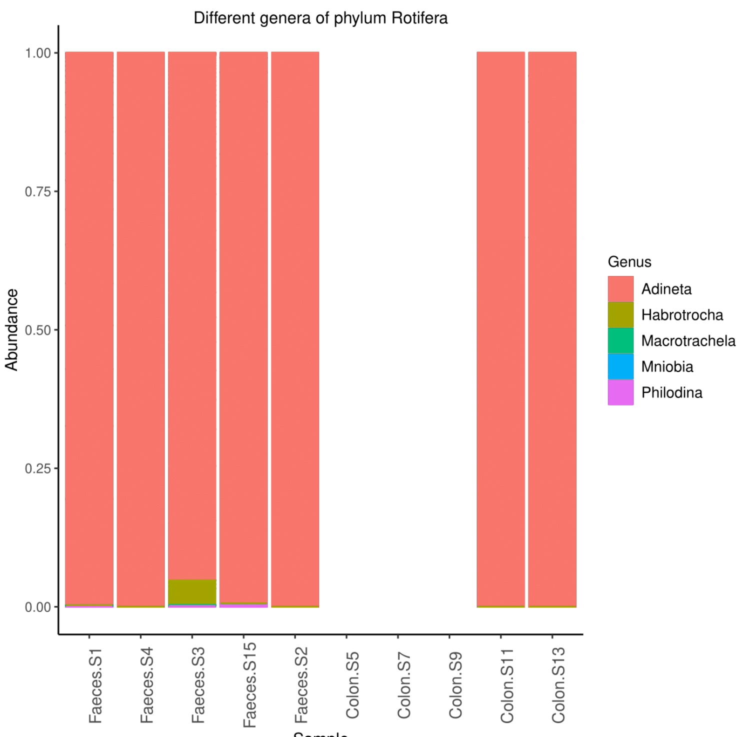


Figure 5-8. Comparison of the proportion of Rotifera genera identified through HTS (high throughput sequencing) and the sample source (hedgehog faecal and colon content) using rAnacapa.

The observed diversity had a greater range for faeces (12–97 ASVs) compared to colon (8–26 ASVs) (Figure 5-9 and Figure 5-10) samples. Conversely, the Shannon diversity shows a greater range for colon (0.05–2.13) compared to faeces (1.61–2.57) (Figure 5-11 and Figure 5-12) samples. The output from both these models shows the faecal samples with both a wider range and higher abundance of ASVs observed. The test results for the alpha (Table 5-7 and Table 5-8) and beta diversities (Table 5-9 and Table 5-10) are reported below.

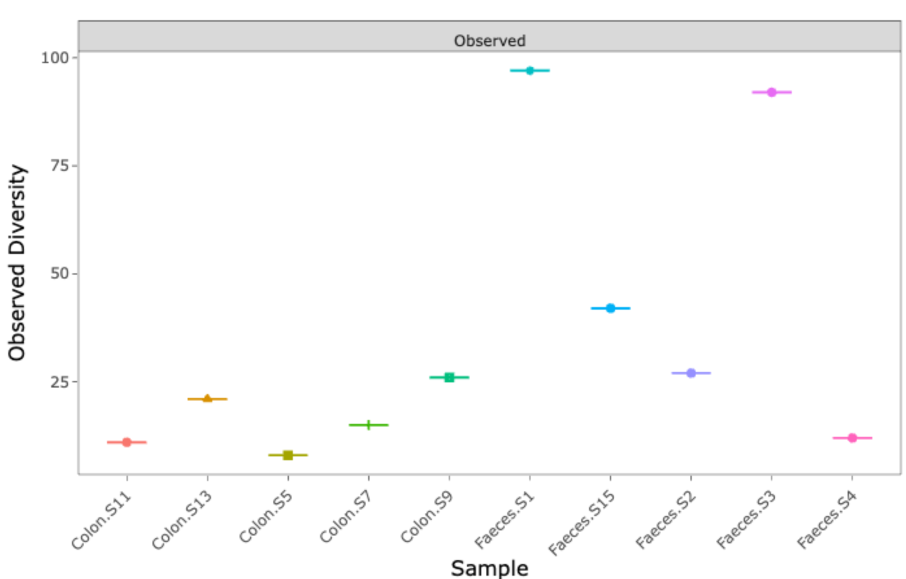


Figure 5-9. Visualisation of the observed diversity for the taxonomic identifications in each sample analysed for the diet of hedgehogs.

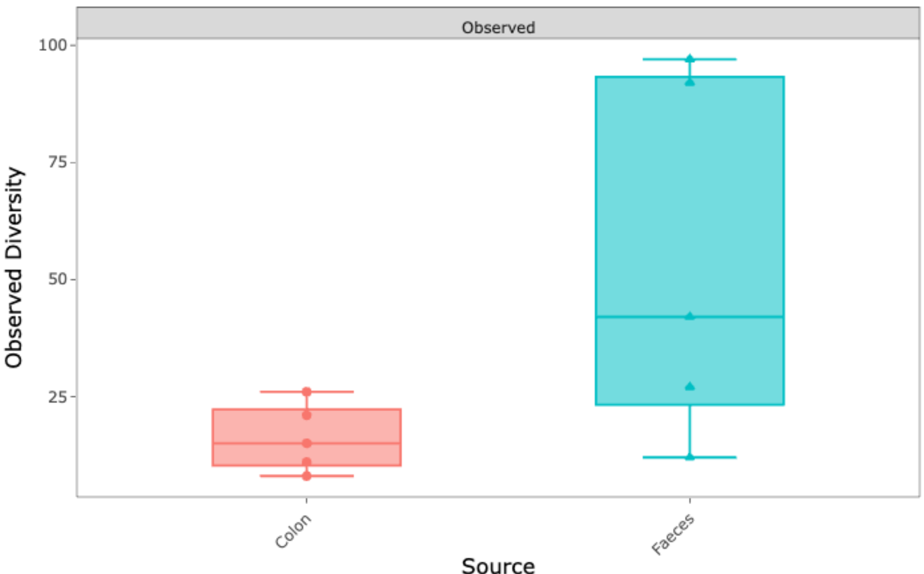


Figure 5-10. The distribution of the observed diversity for the taxonomic identifications in each of the sample types analysed for the diet of hedgehogs.

Table 5-7. Statistical test results for the observed diversity

term	df	sumsq	meansq
Sample	8.0000	1774.2222	221.7778

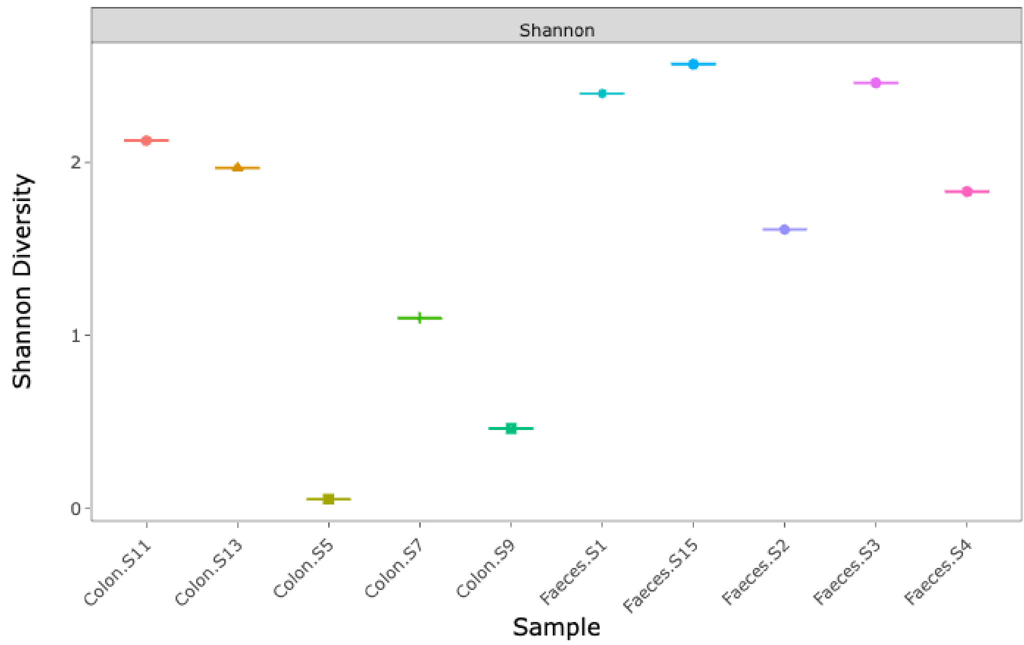


Figure 5-11. Visualisation of the Shannon diversity for the taxonomic identifications in each sample analysed for the diet of hedgehogs.

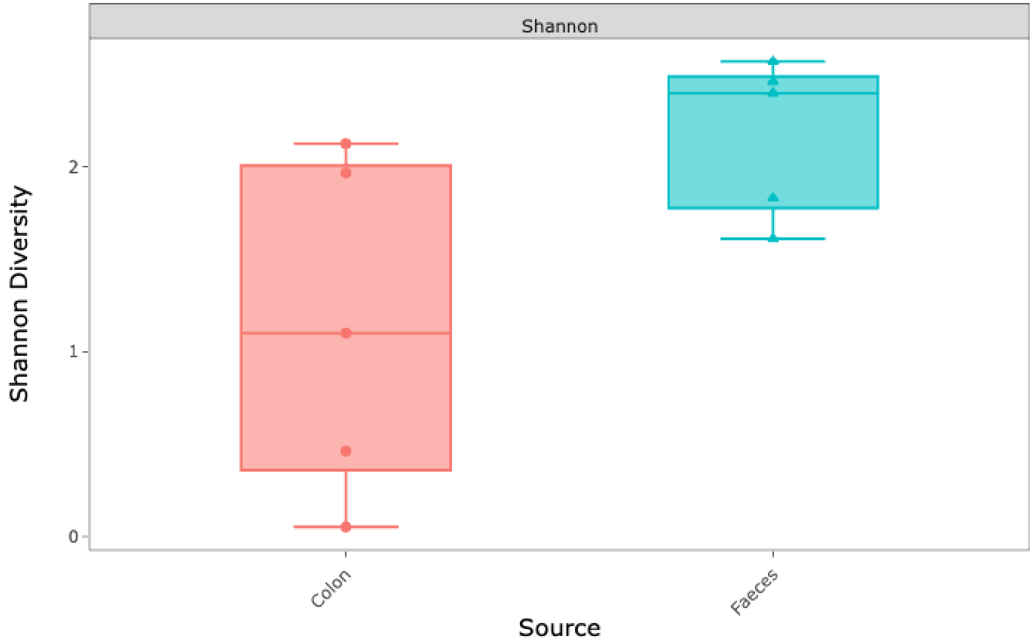


Figure 5-12. The distribution of the Shannon diversity for the taxonomic identifications in each of the sample types analysed for the diet of hedgehogs.

Table 5-8. Statistical test results for the Shannon diversity.

term	df	sumsq	meansq
Sample	8.0000	6.4131	0.8016

While the Jaccard Index of dissimilarity did not show a conclusive grouping of samples based on samples (Figure 5-13, Table 5-9; p-Value = 1.00), some level of grouping based on source type was observed (Figure 5-14, Table 5-10; p-value = 0.01100).

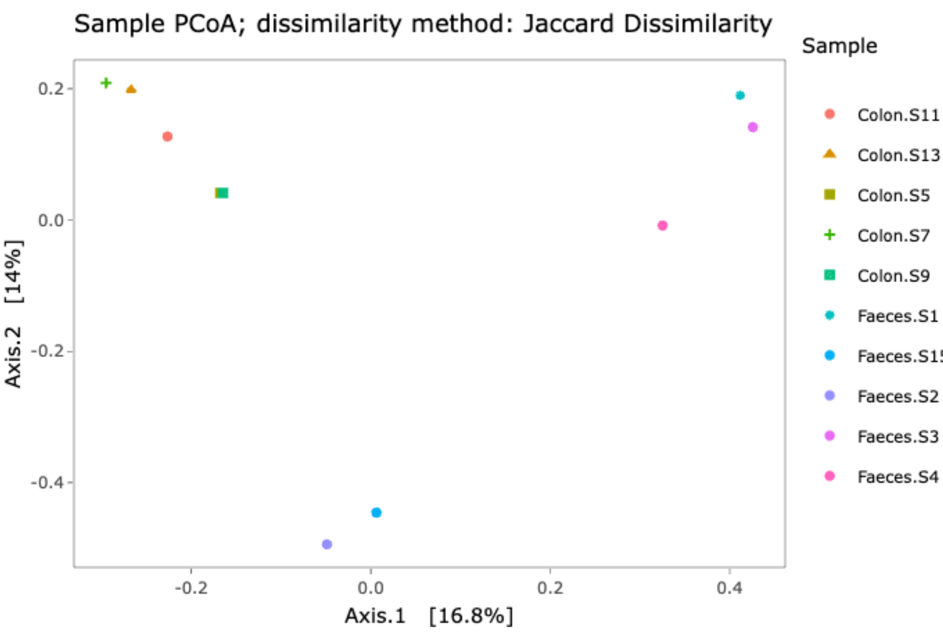


Figure 5-13. Comparison of the Jaccard dissimilarity of the taxonomic identifications in the individual samples for the diet of hedgehogs. The PCoA plot shows 30.8% of the total variance between each sample.

Table 5-9. Multivariable ANOVA table for the Jay-Curtis sample analysis.

term	df	SumsOfSqs	MeanSqs	F.Model	R2	p.value
sample	8.00000	3.68064	0.46008	0.00000	1.00000	1.00000
Residuals	0.00000	0.00000	Inf	NA	0.00000	NA
Total	8.00000	3.68064	NA	NA	1.00000	NA

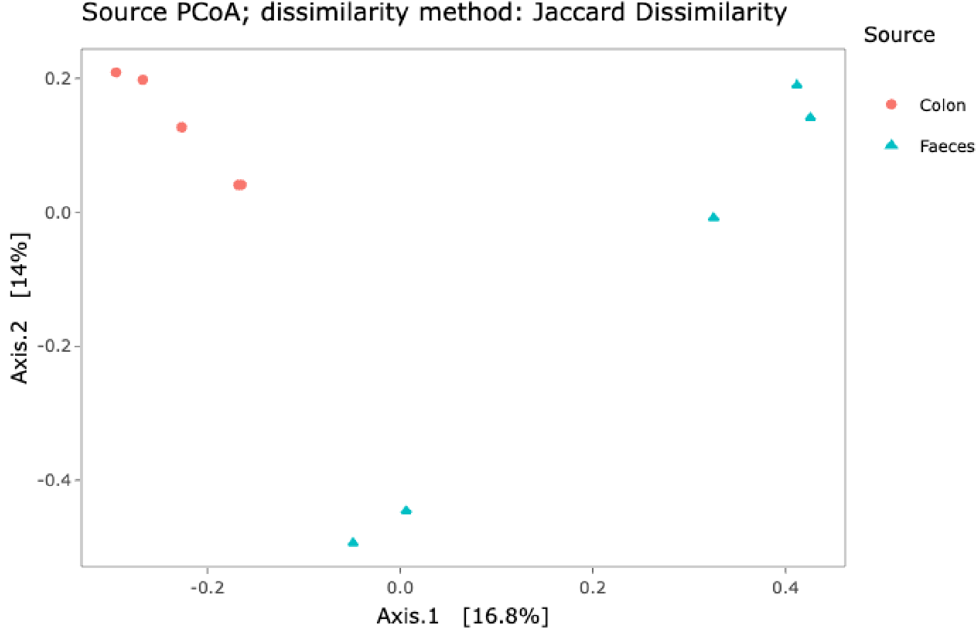


Figure 5-14. Comparison of the Jaccard dissimilarity of the taxonomic identifications in each sample type for the diet of hedgehogs. The PCoA plot shows 30.8% of the total variance between the sample sources.

Table 5-10. Multivariable ANOVA table for the Jay-Curtis Source analysis

term	df	SumsOfSqs	MeanSqs	F.Model	R2	p.value
source	1.00000	0.71290	0.71290	1.67782	0.19335	0.01100
Residuals	7.00000	2.97428	0.42490	NA	0.80665	NA
Total	8.00000	3.68718	NA	NA	1.00000	NA

5.5 Discussion

This chapter used metabarcoding to analyse the diet of the European hedgehog from faecal content collected non-invasively from Kaitorete Spit and colon content collected from hedgehogs caught and euthanized as part of existing predator control on the same site of Kaitorete Spit. Furthermore, the suitability of these metabarcoding methods for the analysis of hedgehog dietary preference from both colon and faecal samples were evaluated. The dietary data can be used alongside other implemented monitoring methods on Kaitorete Spit after the removal of mammalian pests to assess the wider impact of hedgehogs on the native ecosystem.

The consensus in all metabarcoding studies is that the accuracy of the results reflects the available reference databases against which the generated sequences are searched to find consensus taxonomic rank (Collins et al. 2021; Specchia et al. 2020). To account for the variable availability of sequences, I summarised the results on the genus and family (only for Arthropoda) levels, rather than species, because species assignment based on the public databases are unreliable at lower levels (Verkuil et al. 2022) when a local reference database is not available. My study shows the presence of a considerable level of heterogeneity in the prey items, not only between different sources (faeces and colon) but also within each source. As such, it is necessary to take these sources of variation into account to obtain a better resolution of an organism's feeding preferences.

The diet of hedgehogs in this study consisted of a high abundance of Arthropoda in the colon contents while faecal samples showed a greater diversity of the other phyla, such as Mollusca, and Annelida. These results are consistent with earlier studies on hedgehog diet by Nottingham et al. (2019) and Yalden (1976) that reported Arthropoda and Annelida are major components of the hedgehog diet. From this sample size, it cannot be determined if the observed difference is meaningful; however, this thesis does show individual variation between hedgehogs and that there is no uniform diet.

The Arthropoda family Carabidae (Lepidoptera) was most common in colon content in three samples while the most common Arthropoda family in faeces was Trigonidiidae (Orthoptera) in two samples. Interestingly, sample Colon.S5 only consisted of Arthropoda belonging to the Crambidae family. This high abundance in a single colon may be caused by the hedgehog raiding a nest/egg site (Moss 1999) prior to sample collection before the hedgehog defecated. Lepidoptera larvae have been abundant in 41% of stomach samples in past studies of hedgehog diets (Hendra 1999). With current methodologies, metabarcoding cannot distinguish between adult and juvenile stages (Thomsen & Willerslev 2015), therefore, it is an assumption that this hedgehog (Colon.S5) may have feed on a cluster of crambid caterpillars rather than catching multiple flying adults.

Annelida was detected in all faecal samples and, surprisingly, in only one colon sample (Colon.S13). Three genera (*Apporrectodea* Orley, 1885, *Dasybrachus* Grube, 1850, and *Lumbricus* Linnaeus, 1758) were abundant in at least one faecal sample, while *Aporrectodea* dominated the colon sample. *Aporrectodea* contains some earthworms that are abundant in grassland and agricultural systems in New Zealand (Perez 2009). Mollusca was only detected in the faecal samples represented by 2 slug genera (*Arion* and *Deroceras*) (Figure 5-6). Both earthworms and slugs have been observed in hedgehog stomachs elsewhere at a high frequency of 43% and 23% respectively (Nottingham et al. 2019). There was a diverse range of Nematoda genera detected in all faecal samples and three colon samples (Figure 5-7). Rotifera were also detected in all faecal samples and two colon samples with both sample types being dominated by the genus Adineta (Figure 5-8). Both phyla are unlikely to be intentionally consumed by hedgehogs due to the organism's small size and may be parasites of hedgehogs (Gaglio et al. 2010), secondary predation, which is prey of the consumed prey, or environmental contamination from the soil or water (King et al. 2008). There are four known nematode parasites of hedgehogs *Crenosoma striatum* Zeder, 1800, *Eucoleus aerophilus* (Creplin, 1839), *Capillaria erinaceid* Rudolphi, 1819, and *Capillaria ovoreticulata* Laubmeier, 1985 (Gaglio et al. 2010). *Crenosoma striatum* was detected in my study and is also known to be a common parasitic nematode specific to the European hedgehog (Allen et al. 2020).

Allen et al. (2020) monitored hedgehog presence in Europe by sampling *C. striatum* in the nematodes intermediate lifecycle host (gastropods). The study showed potential for detecting hedgehogs using the nematode in areas with low hedgehog abundance (Allen et al. 2020). A high abundance of parasites in hedgehogs may factor into the declines observed in European environments from illnesses such as lungworm (Gaglio et al. 2010). Members of Arthropoda were predominantly identified from the colon content and only two of the faeces failed to show any presence of arthropod prey items, which highlights the importance of Arthropods in the hedgehog diet. Similarly, members of Mollusca were only detected in three of the faecal samples.

Overall, these results of this study emphasise that studies aimed at reconstructing the diet of an animal need to consider different sources of variation prior to any conclusive inference that can be applied in ecosystem management planning. Reconstructions of diet based on multiple sources and multiple specimens per source can help ecosystem managers obtain a better picture of an organism's dietary preferences.

5.5.1 The critical role of a local reference database

In my study, several ASVs from metabarcoding were matched to the local reference library constructed based on traditional barcoding (using sangar sequencing) of individuals from Kaitorete Spit (Table 5-3). Among these matches, seven ASVs from colon samples showed more than 96%

similarity to the sequences in the reference library. In the absence of such locally generated reference libraries, those ASVs could have matched species never reported from the area, resulting in poor taxonomic rank assignment (Specchia et al. 2020). Interestingly, one ASV with a large abundance (n=114,439) was for *Achyra affinitalis* (Lederer, 1863) (Lepidoptera, cotton web spinner) with 60% similarity. This is interesting since the low grade at species and order level indicates this match could only provide a taxonomic match at higher taxonomic rank (e.g., phylum: Arthropoda). This poor match indicates that the match may not be correct as the sequence has been identified as the closest match from reference sequences available in this analysis (Verkuil et al. 2022). This result highlights the need for the elaboration of a curated local reference library prior to interpreting metabarcoding results.

Among the three species of lepidoptera that were identified from colon content, which were *Orocrambus vittellus* Doubleday, 1943 (a NZ grass moth) *A. affinitalis* (Cotton web spinner) and *Eudonia submarginalis* Walker, 1863 (a NZ grass moth), two species (*O. vittellus* and *E. submarginalis*) are endemic and one d (*A. affinitalis*) is introduced (https://biotanz.landcareresearch.co.nz). Similarly, the small blowfly *Calliphora vicina* Robineau-Desvoidy, 1830, was identified from the colon samples. *Calliphora vicina* was accidentally introduced into New Zealand and is useful in forensic entomology to determine the time of death (Arnaldos et al. 2005).

When molecular methods are used for diet analysis, there is potential non-target DNA, mainly from species that were not consumed as part of the diet, to be identified (King et al. 2008). For the colon content, compared to faecal samples, DNA that was extracted directly from the hedgehogs under a controlled laboratory set up could contain less non-target DNA. It is more likely that dietary items present in colon content is the actual species that were consumed by the hedgehog. In the faecal samples, the presence of *Bobilla* sp. Otte & Alexander, 1983, a small cricket and *Phalangium opilio* Linnaeus, 1758, the European harvestman is important. *Bobilla* species have been reported as part of hedgehog diet in previous morphological studies where hedgehogs are known predate orthopteran species with presence in 16% of faecal samples (Jones & Norbury 2011) and 20% of stomach samples (Nottingham et al. 2019). Opiliones (harvestmen) have also been detected in the diet of hedgehogs in previous studies with presence in <5% of stomach contents analysis by Nottingham et al. (2019).

The local reference library used in this study was primarily established to monitor the New Zealand endemic invertebrates that are found on Kaitorete Spit, such as species of *Pericoptus*. From the genus, *Pericoptus frontalis* Broun, 1904 has been found in morphological studies of hedgehog diets in 16% of faecal samples (Jones & Norbury 2011). Although *P. frontalis* itself is only found in Central Otago (Jones & Norbury 2011), the previous evidence that hedgehogs predate this species of *Pericoptus* shows that all these large scarab species are at risk. I did not detect any *Pericoptus* in my

study; however, I only had a small sample of a snapshot in time and the risk from hedgehogs for species not detected in this study should not be assumed. Insect rarity may contribute to the lack of rare insects being seen in the diet, not that rare species are not being eaten (Moss 1999).

5.5.2 General comments

The high abundance of a single taxon in some samples (e.g., FAECES.S4, FAECES.S2, COLON.S5) shows that hedgehogs may be taking advantage of temporarily available food resources, as seen during visual dietary analysis by Campbell (1973) during high abundances of grass grub beetles and Jones and Norbury (2011) with a large occurrence of Mollusca eggs in faecal samples.

In metabarcoding studies, it is standard protocol that all ASV singletons are filtered out, since they are more likely to represent sequencing errors or the presence of non-target DNA; however, singletons are not guaranteed to be wrong. For instance, a closer inspection of the data shows a singleton that matches the common grass skink (*Oligosoma polychroma* (Patterson & Daugherty, 1990)) and is a species of conservation importance. I bring attention to this particular species because of the importance of reptiles in New Zealand and the endangered status of many of the native species that require urgent attention (Hitchmough et al. 2021). Numerous morphological studies have shown evidence of hedgehog predation on lizards (Fountain et al. 2013) with detection in 14% of faecal samples and, in a rare case, one faecal sample contained 10 McCann's skink feet indicating at least three lizards were consumed in one feeding period (Jones & Norbury 2011). Lizards are usually observed in small proportions during studies (2% of hedgehog stomachs analysed (Nottingham et al. 2019)). Hedgehogs still have the potential to harm geographically restricted species and the lack of lizards, or any other species in this thesis, should not be taken as conclusive evidence that hedgehogs do not predate on those species.

I sampled both faecal and colon content to determine if one source was more suitable for molecular analysis. Earlier studies showed that using stomach or faeces did not affect the successful amplification of frog DNA in hedgehog samples (Egeter et al. 2015). In the same way, stomach or faeces do not identify significantly different dietary in morphological studies (Berry 1999; Brockie 1959; Campbell 1973); however, faecal samples are at a significantly higher risk of DNA degradation from environmental exposure. The success of PCR reactions has been shown to decline with an increase in environmental exposure days, especially after seven days when success dropped from 63% to 2% at 14 days (Woodruff et al. 2015). To address the issue of DNA degradation, the primer pair used in this study targets a short fragment of DNA (~300 bp) therefore concerns with small DNA fragments in degraded (Elbrecht et al. 2019) and digested (King et al. 2008) DNA should be of little significance.

In addition to the DNA degradation and fragmentation, both faecal and colon samples have the added difficulty of the potential for inhibitory compounds that may be present in the stomach (Buckwalter et al. 2014). Several methods have been suggested to overcome this issue, including in-lab techniques such as adding BSA (bovine serum albumin) to the PCR reaction mix (Juen & Traugott 2006) to minimise the risk of PCR failures. Despite these concerns, the results of my study show that DNA from both hedgehog faecal and colon samples can successfully get amplified and identify for a wide range of taxa.

5.5.3 Limitations and future directions of the study

This study shows the importance of having a local barcode library for taxonomic rank assignment. In the absence of a reference library, the bioinformatic pipeline reports the closest match to the unknown sequences, which may be a species not found at the study site (Collins et al. 2021). In some cases, these matches only provide information on the dietary preference of an organism at the higher taxonomic levels (order) that is not optimal for ecosystem management plans. Given the diverse ecosystem across New Zealand, I expect this bias to be more obvious in under-studied geographical locations where little is known about the diversity of the ecological communities that inhabit those areas. Kaitorete Spit had few sequenced and databased species prior to my study, other than well-known previously researched species, most often invasive or introduced species.

Due to time restraints, I did not undertake any morphological analysis of the faecal and colon contents prior to molecular analysis; however, this should not change the validity of my results as previous studies have shown molecular approaches to work more reliably than morphological analysis (Egeter et al. 2019). It is worth noting that the molecular reconstruction of the diet of hedgehogs in this study represents a single snapshot of the prey items consumed by the hedgehogs prior to sample collection. As such, the result of this study, and other similar studies, need to be interpreted within this limitation. There is no doubt that future studies that investigate dietary preferences across multiple individuals also through the time can provide a better picture of the dietary preference of the species. Future studies can also compare dietary preferences between hedgehogs in their native habitats in comparison to their invasive habitats to better understand the changes in the feeding behaviour of this species.

Metabarcoding reconstruction of the diet, while providing an unparalleled level of information about an organism's dietary preferences, is sensitive to well-documented methodological artifacts, such as PCR biases which is preferential amplification of some taxa compared to others or bioinformatic limitations that arise from taxonomic assignment (Serite et al. 2022). While recent developments in high throughput sequencing and the availability of more sensitive biochemical assays aim to minimise such limitations, I recommend future studies can benefit substantially from a multi-

disciplinary approach that gathers information from direct observation, identification of the prey item in body excretion, and isotope analysis in combination with metabarcoding. Basing management and conservation of invasive and endangered species on information from a single method is not recommended because the well-documented biases in all these methodologies can result in poor implementation of such management plans.

The next step for this study would be to determine the sequences of additional endemic species found at Kaitorete Spit and search for them within the existing sequence database from both faecal and colon content. It was not feasible to generate barcodes for sequencing from all species in the study area, as part of Chapter 4 of this thesis, due to time and budget constraints. Species such as Kupe's moth that are of significance to local management should be of high importance now that the molecular methods are shown to work. I also recommend the implementation and testing of the applied molecular methods, which have been shown in this study to be successful, to a wider population of hedgehogs from Kaitorete Spit across micro-habitats on the peninsula. Other studies have used GPS trackers to monitor hedgehog movement and assign faeces to individual hedgehogs (Jones & Norbury 2011). Tracking individuals is an additional step that researchers could implement in more locations to monitor the hedgehog diet and any trend shown by an individual, within a population and seasonally.

The diversity of the hedgehog's feeding behaviours makes the predator a major threat to the local ecosystem and, in agreement with Jones (2019), I recommend further research aims at determining effective and standardized methods to control hedgehogs to minimise the threat observed in my study.

5.5.4 Conclusion

My research provides conclusive evidence for the presence of various invertebrates in the European hedgehog diet, which is based on high throughout sequencing of both faecal and colon contents. This study has also highlighted the diversity of the hedgehog feeding behaviours that makes this predator a major threat to multiple native taxa.

The dietary results add strength to ongoing projects to control hedgehogs in New Zealand and to prevent detrimental declines in endangered endemic species because of hedgehog predation. This study has also shown that even in a small geographical location, such as Kaitorete Spit, the diet of hedgehogs is diverse and dietary preference varies significantly between individuals even when samples are collected at the same time. The molecular methods used in my study have provided a complementary method to reconstruct the hedgehog dietary items from soft bodied prey species that are well documented to be overlooked during morphological studies. My study represents the

first stepping stone to better understand the complex diet of hedgehogs and highlights the importance of better understanding of an invasive species diet to properly understand their negative impact on the local ecosystem.

5.6 References

Allen, S., Greig, C., Rowson, B., Gasser, R. B., Jabbar, A., Morelli, S., Morgan, E. R., Wood, M., & Forman, D. (2020). DNA footprints: using parasites to detect elusive animals, proof of principle in hedgehogs. *Animals, 10*(8), 1420. https://doi.org/https://doi.org/10.3390/ani10081420

Arnaldos, M. I., García, M. D., Romera, E., Presa, J. J., & Luna, A. (2005). Estimation of postmortem interval in real cases based on experimentally obtained entomological evidence. *Forensic Science International, 149*(1), 57–65. https://doi.org/https://doi.org/10.1016/j.forsciint.2004.04.087

Berry, C. (1999). Potential interaction of hedgehogs with North Island brown kiwi at Boundary Stream Mainland Island. *Conservation Advisory Science Notes No, 268*, Department of Conservation, Wellington.

Brockie, R. E. (1959). Observations on the food of the hedgehog (*Erinaceus europaeus L.*) in New Zealand. *New Zealand Journal of Science, 2*(1), 121–136.

Buckwalter, S. P., Sloan, L. M., Cunningham, S. A., Espy, M. J., Uhl, J. R., Jones, M. F., Vetter, E. A., Mandrekar, J., Cockerill, F. R., 3rd, Pritt, B. S., Patel, R., & Wengenack, N. L. (2014). Inhibition controls for qualitative real-time PCR assays: are they necessary for all specimen matrices? *Journal of Clinical Microbiology, 52*(6), 2139–2143. https://doi.org/10.1128/jcm.03389-13

Bull, P. C. (1968). The smaller placental mammals of Canterbury. *Natural History of Canterbury* (22), 400–402.

Callahan, B. J., McMurdie, P. J., Rosen, M. J., Han, A. W., Johnson, A. J., & Holmes, S. P. (2016). DADA2: High-resolution sample inference from Illumina amplicon data. *Nature Methods, 13*(7), 581–583. https://doi.org/10.1038/nmeth.3869

Campbell, P. A. (1973). The feeding behaviour of the hedgehog (*Erinaceus europaeus* L.) in pasture land in New Zealand. *New Zealand Ecological Society, 20*(1), 35–40.

Carreon-Martinez, L., Johnson, T. B., Ludsin, S. A., & Heath, D. D. (2011). Utilization of stomach content DNA to determine diet diversity in piscivorous fishes. *Journal of Fish Biology, 78*(4), 1170–1182. https://doi.org/10.1111/j.1095-8649.2011.02925.x

Collins, R. A., Trauzzi, G., Maltby, K. M., Gibson, T. I., Ratcliffe, F. C., Hallam, J., Rainbird, S., Maclaine, J., Henderson, P. A., Sims, D. W., Mariani, S., & Genner, M. J. (2021). Meta-Fish-Lib: A generalised, dynamic DNA reference library pipeline for metabarcoding of fishes. *Journal of Fish Biology, 99*(4), 1446–1454. https://doi.org/https://doi.org/10.1111/jfb.14852

Curd, E. E., Gold, Z., Kandlikar, G. S., Gomer, J., Ogden, M., O'Connell, T., Pipes, L., Schweizer, T. M., Rabichow, L., Lin, M., Shi, B., Barber, P. H., Kraft, N., Wayne, R., & Meyer, R. S. (2019). Anacapa Toolkit: An environmental DNA toolkit for processing multilocus metabarcode datasets. *Methods in Ecology and Evolution, 10*(9), 1469–1475. https://doi.org/https://doi.org/10.1111/2041-210X.13214

Didham, R. K., Barker, G. M., Costall, J. A., Denmead, L. H., Floyd, C. F., & Watts, C. H. (2009). The interactive effects of livestock exclusion and mammalian pest control on the restoration of invertebrate communities in small forest remnants. *New Zealand Journal of Zoology, 36*(2), 135–163. https://doi.org/10.1080/03014220909510148

Dowding, J. E., Elliott, M. J., & Murphy, E. C. (2015). Scats and den contents as indicators of the diet of stoats *(Mustela erminea*) in the Tasman Valley, South Canterbury, New Zealand. *New Zealand Journal of Zoology, 42*(4), 270–282. https://doi.org/10.1080/03014223.2015.1084935

Dowding, J. E., & Murphy, E. C. (2001). The impact of predation by introduced mammals on endemic shorebirds in New Zealand: a conservation perspective. *Biological Conservation, 99*(1), 47–64. https://doi.org/https://doi.org/10.1016/S0006-3207(00)00187-7

Egeter, B., Bishop, P. J., & Robertson, B. C. (2015). Detecting frogs as prey in the diets of introduced mammals: a comparison between morphological and DNA-based diet analyses. *Molecular Ecology Resources, 15*(2), 306–316. https://doi.org/https://doi.org/10.1111/1755-0998.12309

Egeter, B., Roe, C., Peixoto, S., Puppo, P., Easton, L. J., Pinto, J., Bishop, P. J., & Robertson, B. C. (2019). Using molecular diet analysis to inform invasive species management: A case study of introduced rats consuming endemic New Zealand frogs. *Ecology and Evolution, 9*(9), 5032–5048. https://doi.org/https://doi.org/10.1002/ece3.4903

Elbrecht, V., Braukmann, T. W. A., Ivanova, N. V., Prosser, S. W. J., Hajibabaei, M., Wright, M., Zakharov, E. V., Hebert, P. D. N., & Steinke, D. (2019). Validation of COI metabarcoding primers for terrestrial arthropods. *PeerJ, 7*(1), e7745. https://doi.org/10.7717/peerj.7745

Emami-Khoyi, A. (2024, Feb). Reconstruction of the complete mitogenomes of predator and prey from a faecal metagenomic dataset. *Data Brief, 52*, 109830. https://doi.org/10.1016/j.dib.2023.109830

Emami-Khoyi, A., Hartley, D. A., Paterson, A. M., Boren, L. J., Cruickshank, R. H., Ross, J. G., Murphy, E. C., & Else, T.-A. (2016). Identifying prey items from New Zealand fur seal (*Arctocephalus forsteri*) faeces using massive parallel sequencing. *Conservation Genetics Resources, 8*(3), 343–352. https://doi.org/10.1007/s12686-016-0560-9

Ewels, P., Magnusson, M., Lundin, S., & Käller, M. (2016). MultiQC: summarize analysis results for multiple tools and samples in a single report. *Bioinformatics, 32*(19), 3047–3048. https://doi.org/10.1093/bioinformatics/btw354

Ficetola, G. F., Coissac, E., Zundel, S., Riaz, T., Shehzad, W., Bessière, J., Taberlet, P., & Pompanon, F. (2010). An In silico approach for the evaluation of DNA barcodes. *BMC Genomics, 11*(1), 434. https://doi.org/10.1186/1471-2164-11-434

Foster, N. J., Maloney, R. F., Recio, M. R., Seddon, P. J., & van Heezik, Y. (2021). European hedgehogs rear young and enter hibernation in New Zealand's alpine zones. *New Zealand Journal of Ecology, 45*(2), 1–6. https://doi.org/https://doi.org/10.20417/nzjecol.45.52

Fountain, E., Pugh, A., & Bowie, M. H. (2013). *What do predators eat for supper? Burkes Pass Scenic Reserve predator stomach content analysis for 2010–2011*. https://researcharchive.lincoln.ac.nz/handle/10182/6814

Gaglio, G., Allen, S., Bowden, L., Bryant, M., & Morgan, E. R. (2010). Parasites of European hedgehogs (*Erinaceus europaeus*) in Britain: epidemiological study and coprological test evaluation. *European Journal of Wildlife Research, 56*(6), 839–844. https://doi.org/10.1007/s10344-010-0381-1

Gao, X., Lin, H., Revanna, K., & Dong, Q. (2017). A Bayesian taxonomic classification method for 16S rRNA gene sequences with improved species-level accuracy. *BMC Bioinformatics, 18*(1), 247. https://doi.org/10.1186/s12859-017-1670-4

Gordon, A., & Hannon, G. J. (2010). Fastx-toolkit. *FASTQ/A Short-Reads Preprocessing Tools (Unpublished)*. Http://Hannonlab.Cshl.,Edu/Fastx_toolkit

Hebert, P. D., Ratnasingham, S., & De Waard, J. R. (2003). Barcoding animal life: cytochrome c oxidase subunit 1 divergences among closely related species. *Proceedings of the Royal Society of London. Series B: Biological Sciences, 270*(1), S96–S99. https://doi.org/10.1098/rsbl.2003.0025

Hendra, R. (1999). Seasonal abundance patterns and dietary preferences of hedgehogs at Trounson Kauri Park. *Conservation Advisory Science Notes No. 267*, Department of Conservation, Wellington. https://www.doc.govt.nz/documents/science-and-technical/casn267.pdf

Hitchmough, R., Barr, B., Knox, C., Lettink, M., Monks, J. M., Patterson, G. B., Reardon, J. T., Winkel, D. v., Rolfe, J., & Michel, P. (2021). *Conservation status of New Zealand reptiles, 2021* (New Zealand Threat Classification, Issue 35). https://www.doc.govt.nz/globalassets/documents/science-and-technical/nztcs35entire.pdf

Jeske, J. T., & Gallert, C. (2022). Microbiome analysis via OTU and ASV-based pipelines-A comparative interpretation of ecological data in WWTP systems. Bioengineering (Basel), 9(4). https://doi.org/10.3390/bioengineering9040146

Jones, C. (2006). *Impacts of mice and hedgehogs on native forest invertebrates : a pilot study*. Wellington, N.Z. : Science & Technical Publishing, Dept. of Conservation.

Jones, C. (2019). Identifying tools and knowledge gaps to support the control of non-PF2050-targeted small mammalian predators: stakeholder perceptions of priority needs. *LC3567*. https://bioheritage.nz/wp-content/uploads/2019/04/Secondary-preds-gaps.pdf

Jones, C., Garvey, P., Graham, B., & Ross, J. (2021). *Hedgehog (*Erinaceus europaeus*) control tools: Relative attractiveness of potential lures and effects of aperture size on device access*. Department of Pest Management and Conservation. Contract Report: LC3985. https://researcharchive.lincoln.ac.nz/handle/10182/13914

Jones, C., Moss, K., & Sanders, M. (2005). Diet of hedgehogs (*Erinaceus europaeus*) in the upper Waitaki Basin, New Zealand: Implications for conservation. *New Zealand Journal of Ecology, 29*(1), 29–35. https://newzealandecology.org/nzje/2244

Jones, C., & Norbury, G. (2011). Feeding selectivity of introduced hedgehogs (*Erinaceus europaeus*) in a dryland habitat, South Island, New Zealand. *Acta Theriologica, 56*(1), 45–51. https://doi.org/10.1007/s13364-010-0009-6

Juen, A., & Traugott, M. (2006). Amplification facilitators and multiplex PCR: Tools to overcome PCR-inhibition in DNA-gut-content analysis of soil-living invertebrates. *Soil Biology and Biochemistry, 38*(7), 1872–1879. https://doi.org/https://doi.org/10.1016/j.soilbio.2005.11.034

Keedwell, R., Maloney, R., & Murray, D. (2002). Predator control for protecting kaki (*Himantopus novaezelandiae*) - Lessons from 20 years of management. *Biological Conservation, 105*(1), 369–374. https://doi.org/10.1016/S0006-3207(01)00220-8

King, R. A., Read, D. S., Traugott, M., & Symondson, W. O. (2008). Molecular analysis of predation: a review of best practice for DNA-based approaches. *Molecular Ecology, 17*(4), 947–963. https://doi.org/10.1111/j.1365-294X.2007.03613.x

Langmead, B., & Salzberg, S. L. (2012). Fast gapped-read alignment with Bowtie 2. *Nature Methods, 9*(4), 357–359. https://doi.org/10.1038/nmeth.1923

Leray, M., Yang, J. Y., Meyer, C. P., Mills, S. C., Agudelo, N., Ranwez, V., Boehm, J. T., & Machida, R. J. (2013). A new versatile primer set targeting a short fragment of the mitochondrial COI region for metabarcoding metazoan diversity: application for characterizing coral reef fish gut contents. *Frontiers in Zoology, 10*(1), 34. https://doi.org/10.1186/1742-9994-10-34

Martin, M. (2011). Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet.journal, 17*, 10–12. http://code.google.com/p/cutadapt/

Matheson, C., Muller, G., Junnila, A., Vernon, K., Miller, M., Greenblatt, C., Hausmann, A., & Schlein, Y. (2008). A PCR method for detection of plant meals from the gut of insects. *Organisms Diversity & Evolution, 7*(1), 294–303. https://doi.org/10.1016/j.ode.2006.09.002

Miller, C. J., & Miller, T. K. (1995). Population dynamics and diet of rodents on Rangitoto Island, New Zealand, including the effect of a 1080 poison operation. *New Zealand Journal of Ecology, 19*(1), 19–27. http://www.jstor.org/stable/24053666

Monterroso, P., Godinho, R., Oliveira, T., Ferreras, P., Kelly, M. J., Morin, D. J., Waits, L. P., Alves, P. C., & Mills, L. S. (2019). Feeding ecological knowledge: the underutilised power of faecal DNA approaches for carnivore diet analysis. *Mammal Review, 49*(2), 97–112. https://doi.org/https://doi.org/10.1111/mam.12144

Moss, K. A. (1999). *Diet, nesting behaviour, and home range size of the European hedgehog (*Erinaceus europaeus*) in the braided riverbeds of the Mackenzie Basin, New Zealand* Unpublished MSc thesis, University of Canterbury, Christchurch, New Zealand.

Murphy, E. C., Clapperton, B. K., Bradfield, P. M. F., & Speed, H. J. (1998). Effects of rat-poisoning operations on abundance and diet of mustelids in New Zealand podocarp forests. *New Zealand Journal of Zoology, 25*(4), 315–328. https://doi.org/10.1080/03014223.1998.9518161

National Pest Control Agencies. (2018). *A8 Pest Mustelids, Monitoring and Control* https://www.bionet.nz/assets/Uploads/A8-Pest-Mustelids-2018-04-LR.pdf

Nottingham, C. M., Glen, A. S., & Stanley, M. C. (2019). Snacks in the city: The diet of hedgehogs in Auckland urban forest fragments. *New Zealand Journal of Ecology, 43*(2). https://doi.org/10.20417/nzjecol.43.24

Ntuli, N. N., Nicastro, K. R., Zardi, G. I., Assis, J., McQuaid, C. D., & Teske, P. R. (2020). Rejection of the genetic implications of the “Abundant Centre Hypothesis” in marine mussels. *Scientific Reports, 10*(1), 604. https://doi.org/10.1038/s41598-020-57474-0

Ogle, C. (1997). *Evidence for the impacts of possums on mistletoes*. https://www.researchgate.net/publication/253639393

Owen, H. J., & Norton, D. A. (1995). The diet of introduced brushtail possums (*Trichosurus vulpecula)* in a low-diversity New Zealand Nothofagus forest and possible implications for conservation

management. *Biological Conservation, 71*(3), 339–345. https://doi.org/10.1016/0006-3207(94)00058-X

Revell, L. J. (2024). phytools 2.0: an updated R ecosystem for phylogenetic comparative methods (and other things). *PeerJ, 12*, e16505. https://doi.org/10.7717/peerj.16505

Rodrigues, M. S., Morelli, K. A., & Jansen, A. M. (2017). Cytochrome c oxidase subunit 1 gene as a DNA barcode for discriminating *Trypanosoma cruzi* DTUs and closely related species. *Parasites & Vectors, 10*(1), 488. https://doi.org/10.1186/s13071-017-2457-1

Sanders, M. D., & Maloney, R. F. (2002). Causes of mortality at nests of ground-nesting birds in the Upper Waitaki Basin, South Island, New Zealand: a 5-year video study. *Biological Conservation, 106*(2), 225–236. https://doi.org/https://doi.org/10.1016/S0006-3207(01)00248-8

Serite, C. P., Emami-Khoyi, A., Ntshudisane, O. K., James, N. C., van Vuuren, B. J., Bodill, T., Cowley, P. D., Whitfield, A. K., & Teske, P. R. (2022). eDNA metabarcoding vs metagenomics: an assessment of dietary competition in two estuarine pipefishes. *bioRxiv*, 2021.2001.2005.425398. https://doi.org/10.1101/2021.01.05.425398

Specchia, V., Tzafesta, E., Marini, G., Scarcella, S., Simona, D. A., & Pinna, M. (2020). Gap analysis for dna barcode reference libraries for aquatic macroinvertebrate species in the Apulia Region (Southeast of Italy). *Journal of Marine Science and Engineering, 8*(7), 538. https://doi.org/https://doi.org/10.3390/jmse8070538

Thomsen, P. F., & Willerslev, E. (2015). Environmental DNA – An emerging tool in conservation for monitoring past and present biodiversity. *Biological Conservation, 183*(1), 4–18. https://doi.org/https://doi.org/10.1016/j.biocon.2014.11.019

Verkuil, Y. I., Nicolaus, M., Ubels, R., Dietz, M. W., Samplonius, J. M., Galema, A., Kiekebos, K., de Knijff, P., & Both, C. (2022). DNA metabarcoding quantifies the relative biomass of arthropod taxa in songbird diets: Validation with camera-recorded diets. *Ecology and Evolution, 12*(5), e8881. https://doi.org/https://doi.org/10.1002/ece3.8881

Vink, C. J., & Kean, J. M. (2013). PCR gut analysis reveals that *Tenuiphantes tenuis* (Araneae: Linyphiidae) is a potentially significant predator of Argentine stem weevil, *Listronotus bonariensis* (Coleoptera: Curculionidae), in New Zealand pastures. *New Zealand Journal of Zoology, 40*(4), 304–313. https://doi.org/10.1080/03014223.2013.794847

Woodruff, S. P., Johnson, T. R., & Waits, L. P. (2015). Evaluating the interaction of faecal pellet deposition rates and DNA degradation rates to optimize sampling design for DNA-based mark–recapture analysis of Sonoran pronghorn. *Molecular Ecology Resources, 15*(4), 843–854. https://doi.org/https://doi.org/10.1111/1755-0998.12362

Yalden, D. W. (1976). The food of the hedgehog in England. *Acta Theriologica, 21*(30), 401–424 https://doi.org/10.4098/at.arch.76-39

Chapter 6
Conclusion

The introduction of mammalian predators into New Zealand and the subsequent negative impact on the endemic ecosystem is a concern to many restoration and management programmes. To successfully control and minimise the harm from an introduced species, there needs to be an understanding of the animal's behaviour and interspecies relationships caused by competition and predation.

In this thesis, I have successfully used two molecular methods to analyse hedgehog diet. First, a species of interest can be targeted using species-specific primers from hedgehog faecal content. Second, high throughput sequencing (HTS)was successfully used to reconstruct the diet of the hedgehog in New Zealand from a pastural and sand-dune environment (Kaitorete Spit). The choice of the method used in future analysis of hedgehog diet depends on the project's goals and budget. For a project with the aim of determining whether hedgehogs predate a species of interest, the first method using species-specific primers is the best suited option, with an estimated cost of $150 NZD per sample, compared to $500 per sample for HTS analysis. A targeted search will provide only results of that species and will not determine the presence of any other species and could have the disadvantage of overlooking species that are of high abundance in hedgehog diet. For a project that analyses the whole diet of hedgehogs in an environment, the HTS methodologies is best suited. Despite HTS methods being comparatively expensive, the output it provides is of an incomparable level of detail that has previously been missed by morphological analysis on the diet of hedgehogs. I have shown that hedgehogs have a high abundance of Arthropoda in their diet, alongside Annelida and Mollusca, that aligns with previous morphological studies in New Zealand and Europe. This high abundance of Arthropoda places most of New Zealand endemic invertebrates at risk of predation from hedgehogs, indicating the high threat that hedgehogs cause.

To support the reconstruction of hedgehog diet, I created a local reference library of invertebrates from Kaitorete Spit. This was an important step to improve the taxonomic rank assignment after HTS sequencing. In any ecosystem with poor species sequencing, the formation of a local reference library is encouraged. My library had 41 sequences and provided 12 additional species matches. A larger local database can only improve the potential for improved taxonomic rank assignment and should be used in future molecular assessments for the reconstruction of diet from hedgehogs and other predatory species. Any reference created, once uploaded onto a public database, contributes to the ever-growing database of known species on a global level. A new project can pick up these sequences and provides a head start for future research.

My study had a small sample size of five colon and five faecal samples. There is no doubt that another trial of the same size will report different abundances of the key taxa identified here, but the overall result is expected to remain the same. More samples are always a benefit to gain a wider understanding of the trends between individuals. From my results, there was no trend observed of feeding preferences of individual hedgehogs; however, the objective of this thesis was to test the methods for reconstructing hedgehog diet, and a future study should design aims for specifically looking at trends between individuals with a larger sample size now the methods have been proven a success.

The topic of this thesis has a conflict with the view point that hedgehogs are harmless mammals and are seen by many members of the public as cute (Potter 1905). Therefore, the desire to control these hedgehog populations should be addressed cautiously. In New Zealand the impact of mammalian predators is well known; however, an international audience could become alarmed. The issue arises when an animal that has been introduced into New Zealand has no natural predators in this new ecosystem. The introduced animal abundance increases, meanwhile, the animal is endangered due to habitat loss and predation pressure in the natural ecosystem. This relationship has been seen with the brushtail possum from Australia (How & Hillcox 2000) and the hedgehog in Europe (Pettett et al. 2018). It should be noted that these species that are detrimental in a non-native environment are not innately harmful, but they are an alien species in an ecosystem that did not evolve to co-exist with. It is important to highlight that the desire to control these species is only for the preservation of vulnerable native species and in their native environments, these 'harmful' species are often in need of their own conservation management. The diet of an invasive species can be used in their native environment to compare dietary changes or inform methodologies to undertake dietary research.

Dietary analysis can provide information on other aspects of the hedgehog's ecology, such as gastrointestinal parasites and behavioural changes in a new environment. Parasites, such as nematodes, can provide information on the species that may have been consequently introduced with hedgehogs and could be researched to determine any flow-on effects of introducing a new parasite into an ecosystem. Many parasites are known to have a life cycle with many hosts, therefore the question of new secondary hosts in a new ecosystem could be analysed in conjunction with hedgehog research.

In conclusion, I believe that the current thesis provides critical proof of concept data for the use of HTS molecular methods for reconstructing the diet of hedgehogs in New Zealand. The data in this thesis can be used to provide ecosystem managers on Kaitorete Spit with key data on what taxa hedgehogs are negatively impacting. Additionally, this thesis can provide a stepping stone for the assessment of hedgehog diet in other ecosystems in New Zealand.

References

How, R. A., & Hillcox, S. J. (2000). Brushtail possum, *Trichosurus vulpecula*, populations in south-western Australia: demography, diet and conservation status. *Wildlife Research, 27*(1), 81–89. https://doi.org/https://doi.org/10.1071/WR98064

Pettett, C. E., Johnson, P. J., Moorhouse, T. P., & Macdonald, D. W. (2018). National predictors of hedgehog *Erinaceus europaeus* distribution and decline in Britain. *Mammal Review, 48*(1), 1–6. https://doi.org/https://doi.org/10.1111/mam.12107

Potter, B. (1905) The Tale of Mrs. Tiggy-Winkle. London, Frederick Warne & Co.

Appendix A

Detecting Mistletoe in Possum Scat

This appendix shows the report I produced in the ECOL699 class at Lincoln University. This paper allowed for me to develop my molecular skills in preparation for undertaking my masters. The aim of this report was to determine if DNA methods could be used to detect mistletoe in possum scat. This report provided the proof-of-concept test, which could be used as an alternative method to monitor mistletoe in the wild. This project was funded by the Christchurch City Council and the results were presented to them and the Banks Peninsula Conservation Trust at the Wildside committee meeting.

A.1 Introduction

New Zealand is has been exposed to a range of introduced mammalian pests since European colonisation, including hedgehogs (*Erinaceus europaeus*)(Fountain, Pugh, & Bowie, 2013), rodents (rats (*Rattus spp.)* and mice (*Mus musculus*))(Marris, 2000), mustelids (stoat (*Mustela erminea*), ferret (*M. furo*), and weasel (*M. erminea*)) (Schlesselmann, O'Donnell, Monks, & Robertson, 2018), possums (*Trichosurus vulpecula*)(Montague, 2000), deer and goats (Chynoweth, Litton, Lepczyk, Hess, & Cordell, 2013). Of these species, possums are widely referred to as one of New Zealand's most harmful mammalian pest (National Pest Control Agencies, 2015). Possums cause damage across all aspects of the ecosystem, from vegetation damage through foliage and fruit browsing (Duncan et al., 2011; Nugent, Fraser, & Sweetapple, 2001) to direct predation of birds' eggs and checks (Dowding & Murphy, 2001; Powlesland, Knegtmans, & Styche, 2000). The endemic fauna and flora of New Zealand evolved without mammalian pests, making them vulnerable to predation and browsing. A plant of interest is the New Zealand mistletoe with this study focusing on white mistletoe (*Tupeia antarctica*) and green mistletoe (*Ileostylus micranthus*). Mistletoes are obligate hemiparasitic plants, where they must attach to host plants and survive on water and nutrients from their hosts. Green and white mistletoe are both found throughout the North and South Island where white mistletoe is found in forest or shrub habitats (NZPCN, n.d.-b), while green mistletoes is found on coastal lowlands rarely in mountain forest habitats (NZPCN, n.d.-a). Neither species is host specific, with a wide range of nearly 300 known host species both native and exotic species (NZPCN, n.d.-a).

Previous monitoring work has looked at white mistletoe in a North Island podocarp forest during a period of increasing possum numbers (Sweetapple, Nugent, Whitford, & Knightbridge, 2002). From other dietary studies and direct observations there is no doubt that possums do eat mistletoes in New Zealand (Ogle, 1997) and young plants are severely limited by possum browsing (Sweetapple et al., 2002). Within one year of an aerial 1080 operation targeting possum control, mistletoe was

detected where it had previously been absent (Sweetapple et al., 2002). Three years after control when the trap-catch index of possum density increased from <3% to 4.6%, mistletoe foliage cover began to decline as possum browse increased. In another study, Owen and Norton (1995) found that in the Haast Valley, western South Island, the mistletoe species *Peraxilla colensoi* was found to not be a major part of possum diet in that area. However, they stated that this may be an atypical year where there was enough alternative food in the subcanopy, therefore possums did not need to climb into the high canopy. Additionally, they stated that the mistletoe may not be uniformly attractive to possums each year due to lack of flowers. These contradicting reports indicate that many factors influence the relationship between possums and mistletoe species.

Despite species variation, sources tend to agree that possums browsing on mistletoe species is detrimental to mistletoe populations (Ogle, 1997) and possums have a strong dietary preference for mistletoe (Sweetapple et al., 2002). Past dietary studies have used a range of methods, evolving as technology has changed. Cuticle analysis was the initial method to analysis gut contents. Cuticle analysis involves further breaking up stomach content with chemical maceration and viewing these under microscope slides (Sweetapple & Nugent, 1998). However, following research established alternative methods as cuticle analysis is time consuming to prepare and not all parts of the possum's diet is detectable this way, such as bird eggs, fruits, and flowers. Alternative methods include point sampling and layer separation. Layer separation analyses the feed layers in the stomach of the possum. Layers occur due to possums generally eating one plant at a time, therefore forming distinguishable layers. Point analysis involves sieving gut content, then identifying large remaining fragments and weighing these. Of these methods, layer separation was shown to be the most successful for determining possum diets (Sweetapple & Nugent, 1998).

As technology has continued to develop over the past 20 years, the use of molecular techniques is becoming more commonly used for diet analysis. Polymerase chain reactions (PCR) are a molecular method that has been used widely to determine the diets of a range of species, including the plant diet of herbivorous invertebrates (Matheson et al., 2008), and spider diets in pasture environments (Vink, McNeill, Winder, Kean, & Phillips, 2011). Previous faecal sample studies have shown that when molecular techniques are used, known diets of species greatly increases as soft bodied, or easily broken-down material is now detectible. For example, for the New Zealand fur seal (*Arctocephalus forsteri*) it was found the known diet only made up about 16% of what was determined by molecular analysis (Emami-Khoyi, 2015). To further build on this new technology, this study aims to use similar techniques to determine if molecular methods are a viable way to detect mistletoe species in possum diets.

The main advantage of detecting mistletoe in possum diets using molecular analysis of their poo will be another method to monitoring mistletoe populations. When in small populations, mistletoe plant can be hard to find in forest ecosystems due to being high in tree canopies. Often the larger the tree the more mistletoe it can host (Kelly, 1998), but large trees are hard to see into the canopy. However, possums being arboreal species are not limited by this and freely browse mistletoe. This is not good for the mistletoe but opens an opportunity for this new monitoring method when possum poo is collected and analysed new mistletoe locations can be detected, or existing ones monitored. This method however, will hopefully only be short lived, as groups such as Pest Free Banks Peninsular have goals that include eradication of possums from areas by as early as 2024 (Pest Free Banks Peninsula, 2020) and the nationwide goal of Predator Free 2050 also aims to eradicate possums by 2050. However, while possums remain, this new method can help to locate remnant mistletoe populations so monitoring of their recovery can continue once possums are eradicated.

A.2 Aims

A.2.1 Design mistletoe specific primers.

A.2.2 Determine if mistletoe can be reliably detected in possum poo using PCR methods.

A.3 Methods

A.3.1 Sample Collection

Mistletoe samples of the two species (white mistletoe (*Tupeia antarctica*) and green mistletoe (*Ileostylus micranthus*)) were provided by Christchurch City Council for feeding trials and lab analysis. Eleven possums used in pen trials at ZIP were fed mistletoe. On the 21st and 22nd of July 2022 four were fed green mistletoe, four fed white mistletoe, and three were fed their regular diet, that did not include mistletoe, with treats of only apples. Poo samples were collected on the 22nd, 23rd and 24th of July 2022 into individual pottles labelled with the date and cage then stored in the freezer until DNA extraction.

A.3.2 Primer Design and testing

Mistletoe specific primers were designed using the plant chloroplast gene ribulose bisphosphate carboxylase (rbcL) as used by Matheson et al. (2008) for detecting plant species in insect guts. The reference mistletoe sequences were downloaded from GenBank (*Tupeia antarctica* GenBank ID: DQ790133.1, *Ileostylus micranthus* GenBank ID: EU544471.1). Primers were designed using the DNA sequencing editing software Sequencher. The set of primers designed were Mistletoe F1 5'-CCGGCTTATTCTAAAACTTTCC-3' and Mistletoe R1 5'-ACCGCTCGACCGTAATTCTTAGC-3'. The specificity of the primers were tested against three non-target plant species of perennial ryegrass (*Lollium perenne*), common wheat (*Triticum aestivum*) and apple (*Malus domestica*).

A.3.3 Lab Analysis

Plant DNA was extracted using a QAIGEN DNeasy Plant Mini Kit. Plant samples were about 5 mm^2, taken from leaves that were previously frozen until extraction took place. No modifications were made to the kit procedure. DNA from the poo samples was extracted using a QAIGEN DNeasy PowerMax soil Kit recommended by past research on seal diets (Emami-Khoyi, 2015; Emami-Khoyi et al., 2016). Poo samples weighed between 3.5g and 6.3g when wet. Modifications were made to the PowerMax soil kit protocol at step 4 where instead of using a vortexing for 10 minutes on high, the sample was placed into a 1600 Mini G SPEX Sample Prep for 10 minutes at 1500 RPM.

PCR amplification was done using an Applied Biosystems Veriti 96 Well Thermal Cycler with a cycling profile of initial denaturation at 94 °C for three minutes, then 35 cycles of 94 °C denaturing for 30 seconds, 48 °C annealing for 30 seconds and extension at 72 °C for one minute followed by final extension at 72 °C for five minutes. When testing the primers design a range of annealing temperatures were used of 48 °C, 49 °C, 50 °C, 51 °C, 51.5 °C, and 52 °C for mistletoe alone and 48 °C, 52 °C, 56 °C, and 60 °C for testing non targets all other parameters remained the same across all PCR

procedures. The PCR products of the two mistletoe species were then sequenced, and no poo samples were sequenced.

A.3.4 Gel reads

Most PCR products were run on 0.5% agarose gels for 15 – 30 minutes at 100 volts to visualise the amplification of mistletoe DNA. One test of non-targets was run on a 4% agarose gel for 3.5 hours at 60 volts to separate small fragments of DNA for a clear reading. The separated fragments were compared to the ladder to determine if the desired DNA was replicated.

A.4 Results

A.4.1 Feeding Trials

All possums successfully browsed on the mistletoe species they were given. Browsing striped most leaves off the bunches (Figure. A.1).

Figure A. 1 White Mistletoe pre feed (Left) and post feed (Right) (Sample 16).

The amount consumed of wet plant mass after both days of feeding was greater for white compared with green mistletoe (Table 1). The total consumed on day one from four possums was 290 g (average 72.5 g) for green mistletoe and 422 g (average 105.5 g) for white mistletoe. The second day had the same pattern with 152 g (38 g average) for green mistletoe and 530 g (average 132.5 g) for white mistletoe.

Table A. 1 Biomass data of the pen trials of possum mistletoe consumption

Sample No.	Mistletoe spp.	Feed Day	Pre-feed Wet Wt (g)	Post-feed Wet Wt (g)	Amount consumed (g)	Total consumed per night for each spp.	Average consumed per night
1	Green	Thursday	166	95	71		
2	Green	Thursday	206	130	76		
3	Green	Thursday	180	138	42		
4	Green	Thursday	197	96	101	290	72.5
5	White	Thursday	213	96	117		
6	White	Thursday	211	76	135		
7	White	Thursday	184	110	74		
8	White	Thursday	186	90	96	422	105.5
9	Green	Friday	192	175	17		
10	Green	Friday	220	183	37		
11	Green	Friday	184	119	65		
12	Green	Friday	176	143	33	152	38
13	White	Friday	215	58	157		
14	White	Friday	195	65	130		
15	White	Friday	192	101	91		
16	White	Friday	198	46	152	530	132.5

A.4.2 PCR analysis

Annealing temperature

All annealing temperatures (48, 49, 50, 51, 51.5, 52, 56, and 60 °C) were successful at amplifying mistletoe DNA. However, 60 °C showed lower intensity.

Sequencing Mistletoe

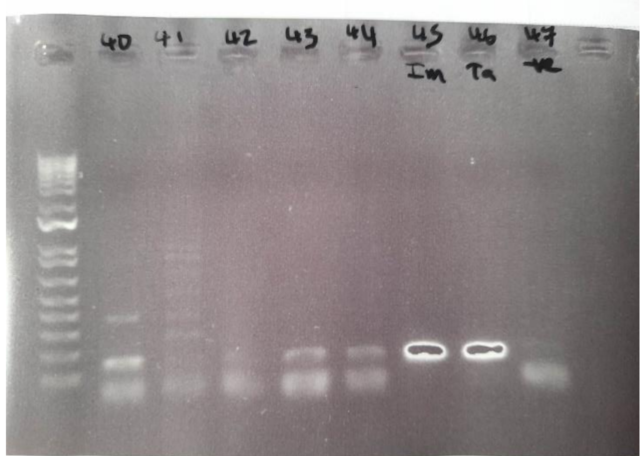
The sequenced DNA strands were 142 and 143 base pairs long (inclusive of the forward and reverse primers) for green and white mistletoe respectively and matched those downloaded from GenBank.

Non-Targets

Testing the primers against non-target plant species showed that perennial ryegrass and common wheat were not amplified, however apple amplification was visualised on the gel indicating amplification from the mistletoe primers.

Possum Poo

Results from the PCR of possum poo showed amplification of something in the scat samples (Figure 2). However due to inconsistencies between the two mistletoe species, and the sample not fed mistletoe (Figure 2; 44), alongside non-target amplification of apple, it cannot be confidently said that it is mistletoe being amplified.



40	*Illeostylus micanthus* fed possum poo
41	*Illeostylus micanthus* fed possum poo
42	*Tupeia antarctica* feed possum poo
43	*Tupeia antarctica* feed possum poo
44	no mistletoe fed possum poo
45	Positive control for *Illeostylus micanthus*
46	Positive control for *Tupeia antarctica*
47	Negative control

Figure A. 2. PCR gel run of Possum Poo with Mistletoe Primer. The sample code and description are to the right of the image.

A.5 Discussion

The feeding trial of white and green mistletoe to possums was successful, showing a readiness of possums to feed on mistletoe, as shown in previous reports (Ogle, 1997; Owen & Norton, 1995; Sweetapple et al., 2002). Incidences of possum browse on green mistletoe has shown to increase with higher trap catches of possums. A 4.6% trap catch showed browsing incidences on up to 75% of plants (Sweetapple et al., 2002). The trend shown with a greater consumption of white mistletoe may be a contributing factor to its rarity on Banks Peninsula in comparison with green mistletoe (A. Evans, personal communication, September 29, 2022) but this will need further research to determine any conclusive relationship.

The results from PCR analysis show that the primers designed in this trial can successfully amplify DNA of white mistletoe (*Tupeia antarctica*) and green mistletoe (*Ileostylus micranthus*). This study has also shown that apple may be an important non-target species that is also partly amplified by this primer. The primers were designed using the plant chloroplast gene ribulose bisphosphate carboxylase (rbcL) (Matheson et al., 2008), therefore the amplified DNA should be plant based and it is unlikely that non-target DNA such as the possum or other non-plant material is being amplified in this process. A similar result was seen when DNA was amplified from the poo of a possum that was not fed mistletoe. This error further indicates that the primer is annealing to plant material not from mistletoe. In response to this finding, future work should aim to design more specific primers by modifying the sequences, or further testing annealing temperatures to exclude non-target DNA such as that from apple. Future work should also look at the amplified DNA sequence extracted from the poo samples and use this to identify what the primer is amplifying. Both these areas for future work were beyond the scope of this study.

There were some limitations to this study that need addressing. Firstly, this study was limited by time and budget, therefore, focused on being a preliminary study to determine if the methods were feasible. Some issues arose, such as amplification of non-target plants and PCR contamination in the lab, indicating that much benefit would come from a continuation of this study to allow for more trial-and-error time. Secondly, only one day of the two days of poo collected were analyses, again due to time restraints, therefore there is plenty of DNA waiting in freezers for the next analysis to see if the second day may have given more time for mistletoe to pass though the possums digestive tract. Finally, the DNA may have been in a low concentration after extractions, therefore for future studies it may also be important to concentrate the extracted DNA through methods such as double PCR.

Overall, this study has successfully designed primers that can amplify mistletoe DNA. However, this study has not been able to confidently detect mistletoe from possum poo samples as there is non-

target amplification. Further work will make steps to produce a method suitable for testing poo samples from the field to determine if this is a reliable method for monitoring wild mistletoe populations. Ultimately, this study has provided initial results into the use of PCR to analyse possum poo as a new method to monitor mistletoe population, however it is acknowledged that there is still much work needed before this method is widely useable.

References

Chynoweth, M. W., Litton, C. M., Lepczyk, C. A., Hess, S. C., & Cordell, S. (2013). Biology and impacts of pacific island invasive species. *Capra hircus*, the feral goat (Mammalia: Bovidae). *Pacific Science, 67*(2), 141-156, 116. Retrieved from https://doi.org/10.2984/67.2.1

Dowding, J. E., & Murphy, E. C. (2001). The impact of predation by introduced mammals on endemic shorebirds in New Zealand: a conservation perspective. *Biological Conservation, 99*(1), 47-64. doi:https://doi.org/10.1016/S0006-3207(00)00187-7

Duncan, R. P., Holland, E. P., Pech, R. P., Barron, M., Nugent, G., & Parkes, J. P. (2011). The relationship between possum density and browse damage on kamahi in New Zealand forests. *Austral Ecology, 36*(7), 858-869. doi:https://doi.org/10.1111/j.1442-9993.2010.02229.x

Emami-Khoyi, A. (2015). *Population and diet of the New Zealand fur seal (Arctocephalus forsteri): molecular approaches.* Lincoln University,

Emami-Khoyi, A., Hartley, D. A., Paterson, A. M., Boren, L. J., Cruickshank, R. H., Ross, J. G., . . . Else, T.-A. (2016). Identifying prey items from New Zealand fur seal (*Arctocephalus forsteri*) faeces using massive parallel sequencing. *Conservation Genetics Resources, 8*(3), 343-352. doi:10.1007/s12686-016-0560-9

Fountain, E., Pugh, A., & Bowie, M. H. (2013). *What do predators eat for supper? Burkes Pass Scenic Reserve predator stomach content analysis for 2010-2011*. Retrieved from https://researcharchive.lincoln.ac.nz/handle/10182/6814

Kelly, D. (1998). *Spatial clumping of Tupeia antarctica at Wainui*. Retrieved from https://bts.nzpcn.org.nz/articles/spatial-clumping-of-tupeia-antarctica-at-wainui/

Marris, J. W. M. (2000). The beetle (Coleoptera) fauna of the Antipodes Islands, with comments on the impact of mice and an annotated checklist of the insect and arachnid fauna. *Journal of the Royal Society of New Zealand, 30*(2), 169-195. doi:10.1080/03014223.2000.9517616

Matheson, C., Muller, G., Junnila, A., Vernon, K., Miller, M., Greenblatt, C., . . . Schlein, Y. (2008). A PCR method for detection of plant meals from the gut of insects. *Organisms Diversity & Evolution, 7*, 294-303. doi:10.1016/j.ode.2006.09.002

Montague, T. L. (2000). *The brushtail possum : biology, impact and management of an introduced marsupial*. Lincoln, N.Z.: Lincoln, N.Z. : Manaaki Whenua Press.

National Pest Control Agencies. (2015). A4.2 Kill Traps. A guide to trap possums, ferrerts, stoats and feral cats using kill traps. Retrieved from https://www.bionet.nz/assets/Uploads/A3-Landowners-2020.pdf

Nugent, G., Fraser, W., & Sweetapple, P. (2001). Top down or bottom up? Comparing the impacts of introduced arboreal possums and 'terrestrial' ruminants on native forests in New Zealand. *Biological Conservation, 99*(1), 65-79. doi:https://doi.org/10.1016/S0006-3207(00)00188-9

NZPCN. (n.d.-a). *Ileostylus micranthus*. Retrieved from https://www.nzpcn.org.nz/flora/species/ileostylus-micranthus/

NZPCN. (n.d.-b). *Tupeia antarctica*. Retrieved from https://www.nzpcn.org.nz/flora/species/tupeia-antarctica/

Ogle, C. (1997). Evidence for the impacts of possums on mistletoes. Retrieved from https://www.researchgate.net/publication/253639393

Owen, H. J., & Norton, D. A. (1995). The diet of introduced brushtail possums (*Trichosurus vulpecula)* in a low-diversity New Zealand Nothofagus forest and possible implications for conservation management. *Biological Conservation, 71*(3), 339-345. doi:10.1016/0006-3207(94)00058-X

Pest Free Banks Peninsula. (2020). *Strategy for a Pest-Free Banks Peninsula* Retrieved from https://pestfreebankspeninsula.org.nz/index.php/the-opportunity/strategy

Powlesland, R. G., Knegtmans, J. W., & Styche, A. (2000). Mortality of North Island tomtits (*Petroica macrocephala toitoi*) caused by aerial 1080 possum control operations, 1997-98, Pureora Forest Park. *New Zealand Journal of Ecology, 24*(2), 161-168. Retrieved from http://www.jstor.org/stable/24054670

Schlesselmann, A.-K. V., O'Donnell, C. F. J., Monks, J. M., & Robertson, B. C. (2018). Clearing islands as refugia for black-fronted tern (*Chlidonias albostriatus*) breeding colonies in braided rivers. *New Zealand Journal of Ecology, 42*(2), 137-148. Retrieved from https://www.jstor.org/stable/26538105

Sweetapple, P. J., & Nugent, G. (1998). Comparison of two techniques for assessing possum (*Trichosurus vulpecula*) diets from stomach contents. *New Zealand Journal of Ecology, 22*(2), 181-188. Retrieved from http://www.jstor.org/stable/24054690

Sweetapple, P. J., Nugent, G., Whitford, J., & Knightbridge, P. I. (2002). Mistletoe (*Tupeia antarctica*) recovery and decline following possum control in a New Zealand forest. *New Zealand Journal of Ecology, 26*(1), 61-71. Retrieved from http://www.jstor.org/stable/24056285

Vink, C., McNeill, M., Winder, L., Kean, J., & Phillips, C. (2011). PCR analyses of gut contents of pasture arthropods. In *Paddock to PCR - Demystifying molecular biology for practical plant protection* (pp. 125-134): New Zealand Plant Protection Society.