Detecting Frogs as Prey in the Diets of Introduced Mammals

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Archey’s frog *Leiopelma archeyi*  

Photo: Bastian Egeter
Abstract

Amphibians are currently the most threatened group of vertebrates, with an estimated 40% of amphibian species currently in danger of extinction. The global spread of introduced fauna has been accepted as a major factor in the decline of amphibians, as native amphibians often have little or no evolutionary history with introduced predators. In particular, introduced small mammals such as rodents pose a major threat, as they are generalist predators that together have an almost global distribution. Wildlife management decisions regarding the control of introduced predators to protect endangered species are often based on predation rates derived from diet studies of the predators concerned. However, many studies have reported difficulties in identifying amphibians as prey in diet analysis, due to the degraded nature of any diagnostic prey components surviving the mastication and digestion processes of the predator. The aim of the research presented in this thesis was to aid global amphibian conservation by assessing the available tools, and developing further tools, to detect frogs as prey of introduced mammals.

I investigated the feeding behaviour of introduced mammals (ship rats *Rattus rattus*, Norway rats *Rattus norvegicus*, house mice *Mus musculus* and hedgehogs *Erinaceus europaeus*) when presented with frogs (southern bell frogs *Litoria raniformis*) as prey and explored the usefulness of two diet analysis methods: traditional morphological analysis and novel DNA-based methods. Using these methods I estimated the impact of ship rats on two endangered New Zealand native frogs (Hochstetter’s frog *Leiopelma hochstetteri* and Archey’s frog *Leiopelma archeyi*).

The results show that, using morphological diet analysis, 11% of stomach and faecal samples collected after small mammals had ingested frogs could be classed as containing frogs as prey, but less than 1% could be identified to species level. Although very small bones can be
used to identify frogs as prey, the odds of successful identification dramatically increase as prey bone length in predator stomach and faecal contents increases.

The use of DNA-based methods increased prey identification (to species level) from less than 1% to 58%. Prey detection periods exceeded known gastrointestinal transit times for the small mammal species concerned, indicating that it is primarily the passage of prey material through the gastrointestinal tract that limits the successful detection of DNA, rather than the degradative effects of ingestion or digestion. The DNA-based methods were validated for field-collected samples and were successful where morphological analysis was not. Both Hochstetter’s frogs and Archey’s frogs were identified in the stomach contents of wild ship rats and estimates of predation rates at the study sites ranged from 0.01–0.9 frogs/ha/night, but it remains unclear whether ship rats alone threaten them with extinction.

This is the first study to investigate the usefulness of DNA-based diet analysis for detecting amphibians as prey, the first to estimate predation rates on New Zealand’s native frogs, and the first to compare stomach versus faecal prey DNA detection over time in any vertebrate. DNA-based diet analyses are highly adaptable and they offer a reliable and cost-effective approach to conservation managers hoping to make assessments of the impacts of introduced fauna on native amphibians, which are necessary to make informed decisions on the implementation of predator control.
Acknowledgements

The completion of this PhD would not have been possible without the friendship, encouragement, expertise, hard work, tolerance, guidance and interest of many people and while I do my best here to thank as many as I can, there are still more I am greatly indebted to and I hope that one day I can return the favour.

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<th>Meaning</th>
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<tbody>
<tr>
<td>$\alpha$</td>
<td>significance level</td>
</tr>
<tr>
<td>$\mu g$</td>
<td>microgram</td>
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<tr>
<td>$\mu l$</td>
<td>microlitre</td>
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<tr>
<td>$\mu M$</td>
<td>micromolar</td>
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<tr>
<td>AIC</td>
<td>akaike information criterion</td>
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<td>ANOVA</td>
<td>analysis of variance</td>
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<td>BLAST</td>
<td>Basic Local Alignment Search Tool</td>
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<tr>
<td>bp</td>
<td>base pair</td>
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<tr>
<td>BSA</td>
<td>bovine albumen serum</td>
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<td>btn</td>
<td>bait tube nights</td>
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<td>CI</td>
<td>confidence interval</td>
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<tr>
<td>cm</td>
<td>centimetre</td>
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<td>corrected trap nights</td>
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<td>df</td>
<td>degrees of freedom</td>
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<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
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<tr>
<td>dNTP</td>
<td>deoxyribonucleotide triphosphate</td>
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<tr>
<td>DOC</td>
<td>Department of Conservation</td>
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<tr>
<td>EF</td>
<td>entire frog</td>
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<tr>
<td>F</td>
<td>ferret</td>
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<td>FF</td>
<td>frog flesh</td>
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<tr>
<td>g</td>
<td>gram</td>
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<tr>
<td>G</td>
<td>gravitational acceleration</td>
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<td>GIT</td>
<td>gastrointestinal tract</td>
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<td>h</td>
<td>hour</td>
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<td>H</td>
<td>hedgehog</td>
</tr>
<tr>
<td>ha</td>
<td>hectare</td>
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<tr>
<td>IUCN</td>
<td>International Union for Conservation of Nature</td>
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<tr>
<td>LNR</td>
<td>laboratory Norway rat</td>
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<td>m</td>
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<td>NGS</td>
<td>next generation sequencing</td>
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<tr>
<td>NH4</td>
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<tr>
<td>NR</td>
<td>(wild) Norway rat</td>
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<tr>
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<td>observed data</td>
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<td>$^\circ C$</td>
<td>degrees Celsius</td>
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<tr>
<td><strong>Abbreviation</strong></td>
<td><strong>Meaning</strong></td>
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<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
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<tr>
<td>RM-ANOVA</td>
<td>repeated measures analysis of variance</td>
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<td>RM-ANCOVA</td>
<td>repeated measures analysis of covariance</td>
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<tr>
<td>RPM</td>
<td>revolutions per minute</td>
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<td>s</td>
<td>second</td>
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<tr>
<td>SD</td>
<td>standard deviation</td>
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<tr>
<td>SE</td>
<td>standard error</td>
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<tr>
<td>SPSS</td>
<td>Statistical Package for the Social Sciences</td>
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<tr>
<td>SR</td>
<td>ship rat</td>
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<td>ST</td>
<td>stoat</td>
</tr>
<tr>
<td>SVL</td>
<td>snout-vent length</td>
</tr>
<tr>
<td>Taq</td>
<td>taq polymerase</td>
</tr>
<tr>
<td>tDP</td>
<td>detection period</td>
</tr>
<tr>
<td>tPA</td>
<td>number of days prey available</td>
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<tr>
<td>tPU</td>
<td>number of nights prey unavailable</td>
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<tr>
<td>U</td>
<td>units</td>
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<tr>
<td>UV</td>
<td>ultraviolet</td>
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Chapter 1 General introduction

1.1 Global amphibian declines

Amphibians are currently the most threatened group of vertebrates, with an estimated 40% of amphibian species currently in danger of extinction (Bishop et al. 2012). Based on the figures from the International Union for Conservation of Nature’s (IUCN) Red List of Threatened Species™, this equates to almost as many Threatened amphibian species as there are Threatened mammals and birds combined (Bishop et al. 2012; IUCN 2013). It was during the First World Congress of Herpetology in September 1989, that it became apparent that amphibian populations in many parts of the world were declining (Wake, 1991; Halliday, 1998; Waldman and Tocher, 1998; Gardner, 2001). In fact, after reviewing the literature and undertaking a number of modeling approaches, researchers realised this was a vastly widespread phenomenon and that declines, while being reported as early as the 1950s in some countries, were occurring at a global scale (Houlahan et al. 2000; Alford et al. 2001).

The decline of amphibians, at both population and species levels, represents the most dramatic example of vertebrate extinction currently taking place (Wake and Vredenburg 2008). This current major loss of biodiversity, which researchers have termed the “sixth mass extinction” (Wake and Vredenburg 2008; Barnosky et al. 2011), has vast ecological, economic, aesthetic and ethical implications (Collins and Crump 2009).

Although the decline of amphibians has been of international concern for more than two decades, the direct causes of these declines are often difficult to identify, or they are difficult to disentangle as they may be acting synergistically (Alford and Richards 1999; Alford et al. 2001; Stuart et al. 2004). This is exacerbated by the many amphibian traits that can make them difficult to study, in comparison to other vertebrates, such as: being relatively small
animals, often having widely fluctuating populations, having a distinctly different larval and adult stage, living in both terrestrial and aquatic habitats, migrating and varying habitat use seasonally, spending long periods in states of torpor or estivation, spending a high portion of time in refugia difficult for researchers to observe (e.g. under benthic mud, under deep rock piles), emerging from refugia only ephemerally (on both seasonal and daily timescales), often being nocturnal, and often favouring humid, wet or rainy conditions, which can hamper field studies (see Heyer et al. 1994 and references therein for detailed discussion on amphibian measuring and monitoring methods). These and other factors have led to an alarming number of species (23 %) being placed in the IUCN’s Data Deficient category, which is much higher than for the other comprehensively studied vertebrate groups, birds and mammals (Bishop et al. 2012).

Proposed causes of amphibian decline include habitat destruction, pollution, exotic species introductions, disease, overexploitation, climate change and UV radiation (Adams 1999; Lau et al. 1999; Blaustein and Belden 2003; Dodd and Smith 2003; Green 2003; Jensen and Camp 2003; Kats and Ferrer 2003; Stuart et al. 2004; Thomas et al. 2004; Beebee and Griffiths 2005; Hayes et al. 2006; Mendelson et al. 2006; Reading 2007; Wake 2007; Lips et al. 2008; Rohr et al. 2008; Ribeiro et al. 2009). Where causes of decline can be identified, they are generally very difficult to remedy, and countering amphibian declines is one of the greatest conservation challenges of the century (Bishop et al. 2012).

1.2 The impact of introduced fauna on amphibians

The breakdown of major geographic barriers, via infrastructure developed by humans, is transforming Earth’s biota through the intentional or accidental introduction of new species, which alters the composition and ecology of biological communities (Wilson 1999; Mooney and Hobbs 2000; Davis 2003). Introduced fauna are considered one of the most important threats to global biological diversity – second only to habitat destruction (Earth Summit, Rio
Convention 1992; http://www.biodiv.org; Vitousek et al. 1996; Park 2004). Regarding amphibians specifically, introduced fauna are ranked as the third most important factor after habitat modification and pollution (Chanson et al. 2008). Native amphibians often have little or no evolutionary history with introduced predators and as a result can exhibit naive responses that are not well suited for survival (Diamond and Case 1986; Gillespie 2001; Kats and Ferrer 2003). The effect of introduced predators is predicted to be most acute in island ecosystems (Schoener and Spiller 1996; Courchamp et al. 2003; Ahola et al. 2006), particularly on endemic island species (Diamond and Veitch 1981), and their impact can be manifested through herbivory, predation, competition, vectors of disease or parasites, hybridisation, habitat alteration, altering behaviour through causing stress responses on prey species, and acting as prey for other predators (Ebenhard 1988; Boonstra et al. 1998; Kats and Dill 1998; Collins and Storfer 2003; Kats and Ferrer 2003; Park 2004).

There have been many studies showing a negative correlation between the introduction of novel predators and native amphibian populations (reviewed by Kats and Ferrer 2003). Fish appear to be the most geographically widespread of the introduced predator groups affecting amphibians, particularly affecting egg and larvae survival (Stebbins 1997; Kats and Ferrer 2003), hence the majority of studies have focused on introduced fish (or other introduced amphibians) as agents of decline. However, Ahola et al. (2006) noted that the impact of introduced predators (fish or otherwise) on adult frogs was largely unknown.

Toledo et al. (2007) carried out a review of post-metamorphic anurans as prey of vertebrates (defined by percentage of reports attributed to each predator group, and irrespective of being native or introduced). I used the supporting material (predator species, prey species and cited reference) from Toledo et al. (2007), along with the known global distribution of the predator in each case (IUCN 2013) to examine the proportion of reports involving introduced predators. They identified the primary predatory groups as Serpentes (45 %), Mammalia (c.
16\%), Aves (c. 15\%), Anura (c. 12\%) and Actinopterygii (4\%) and noted that all the main
groups of vertebrates were represented as predators of anurans to some extent (Toledo et al.
2007). Reordering these groups based on the proportion (%) of predators classed as
introduced, I found the following results: Actinopterygii (55.6\%), Mammalia (5.3\%), Anura
(3.5\%), Aves (2.7\%) and Serpentes (0\%). This shows that although relatively few of the
predation reports were attributed to Actinopterygii, over half were by introduced species.
Conversely, the most reported group of anuran predators (Serpentes) was comprised entirely
of native species. The remaining three taxa (Mammalia, Anura and Aves) included similar
proportions of introduced species (see also Figure 1.1).

It is important to note that the review only included reports where prey had been identified to
species level (Toledo et al. 2007). It is often very difficult to identify anurans as prey (see
Section 1.5.1 and Chapter 3 of this thesis), and this could have resulted in a large bias of
reports on predators from which prey can be more easily identified (e.g. predator groups that
ingest frogs whole). Furthermore, it was largely limited to reports from Herpetological
Review, and as such may have included a higher proportion of reports of the prey of reptiles
and amphibians than that of other taxa. Nonetheless, the apparent difference in ranking of the
importance of predator taxa impacting anurans is striking, when incorporating their status as
introduced.

1.3 New Zealand frogs

New Zealand’s native amphibian fauna consists of four extant frog species, all belonging to
the genus *Leiopelma* (Leiopelmatidae; Bell 1994a; Newman 1996; Frost et al. 2006; Bishop
et al. 2013). This is an ancient endemic group that has retained unique and primitive
characteristics not found in most other anuran species, such as tail-wagging muscles and free
ribs (both absent in most other species), and 9 amphicoelous (bi-concave) presacral vertebrae
(most anurans have 8 or less and they are generally procoelus – anteriorly concave,
posteriorly convex; Wagner 1934b; Wagner 1934a; de Vos 1938a; de Vos 1938b; Stephenson 1945; Stephenson 1951a; Stephenson 1952; Stephenson 1955a; Moffat 1974; Worthy 1987a; Green and Cannatella 1993; Bell 1994a; Baber et al. 2006).

The group represents the most ancient elements of New Zealand's terrestrial vertebrate fauna (Bell et al. 1985; Bell 1994a), and as such is of high conservation value (Bell et al. 2004b; Bell 2010). Although the genus *Leiopelma* has been grouped with the genus *Ascaphus* (represented by two species in North America) in Leiopematidae (Green et al. 1989; Báez and Basso 1996; Feller and Hedges 1998; Roelants and Bossuyt 2005; San Mauro et al. 2005; Frost et al. 2006), the most recent and comprehensive genetic evidence suggests that *Leiopelma* and *Ascaphus* should be placed in Leiopematidae and Ascaphidae, respectively (Irisarri et al. 2010; Pyron and Wiens 2011). These groups together form a sister group to all other anurans (Frost et al. 2006; Pyron and Wiens 2011), further highlighting their distinctiveness.

Prior to the arrival of humans in the thirteenth century AD (Wilmshurst and Higham 2004; Wilmshurst et al. 2008), three species of *Leiopelma* are known to have existed in New Zealand that have since become extinct (Worthy 1986; Worthy 1987b; Bell 1994a; Newman 1996). From fossils deposited in the Early Miocene (19 – 16 million years ago) a further two species, also extinct, have been recently described (Worthy et al. 2013).

The four extant native frog species are: Archey’s frog (*Leiopelma archeyi*), Hochstetter’s frog (*Leiopelma hochstetteri*), Maud Island frog (*Leiopelma pakeka*) and Hamilton’s frog (*Leiopelma hamiltoni*), and all four are ranked within the top 60 most Evolutionarily Distinct and Globally Endangered (EDGE 2008) amphibians in the world, with Archey’s frog topping that list. Naturally occurring populations of Archey’s and Hochstetter’s frogs are present on the mainland of the North Island, New Zealand, while the Maud Island frog and Hamilton’s frog are restricted to Maud Island and Stephen’s Island respectively (both in the Marlborough
Sounds; Bell 1994a; Bishop et al. 2013). A number of translocations have also taken place so that the Maud Island frog is now also present on Motuara Island (Tocher and Pledger 2005), Long Island (Germano 2006) and at Zealandia Sanctuary (a mammal-proof fenced area near Wellington; Lukis and Bell 2007), while Hamilton’s frog is now also present on Nukuwaiata (Tocher et al. 2006). The majority of these off-shore islands are free of introduced mammalian fauna (mice Mus musculus are present at Zealandia Sanctuary), and so the present study is primarily concerned with Archey’s frog and Hochstetter’s frog – the two remaining mainland species, that occur alongside introduced mammals. However, it must be noted that very recently mice were discovered on Maud Island (DOC 2013). Plans to eradicate the mice are due to come into effect in 2014, but complete eradication can be difficult to attain and the impact this may have on the frogs is unknown. There are presently no known populations of native frogs on the mainland of the South Island of New Zealand. Research has been carried out assessing potential suitable translocation sites (Egeter 2009) and future plans for translocations are being considered (PJ Bishop, pers. comm.).

Archey’s frog

Archey’s frog was previously thought to be restricted to the Coromandel Peninsula (Bell 1982b), but another population was discovered in Whareorino Forest, Northern King Country in 1991 (Thurley and Bell 1994b; Thurley 1996). This is an entirely terrestrial species (Bell 1978a; Bell 1982b; Bell 1982a; Bell et al. 1998) with intracapsular development, rather than free-swimming tadpoles (Stephenson 1951b; Stephenson 1961; Bell and Wassersug 2003). While the overall population at Whareorino Forest appears to be stable (Pledger 2011; Ohmer et al. 2012), the Coromandel Peninsula population appears to have declined dramatically in recent years (Bell et al. 2004a).
**Hochstetter’s frog**

This species is the most widespread of the native frogs, with scattered populations found throughout the northern section of the North Island from Mount Ranginui in the south to Waipu and Great Barrier Island in the north (Bell 1982b; Newman 1996; Gemmell *et al.* 2003; Newman *et al.* 2010; Bishop *et al.* 2013). The most recent discovery of a previously unknown population of Hochstetter’s frog occurred in Maungatautari in 2004 (Baber *et al.* 2006). Studies have strongly indicated that a number of the scattered populations are genetically distinct lineages and possibly even separate species (Green 1994; Gemmell *et al.* 2003; Fouquet *et al.* 2010). The species (or species complex) is semi-aquatic and is usually restricted to cool, rocky streams and seepages in native woodland habitats (McLennan 1985; Newman and Towns 1985; Green and Tessier 1990; Tessier *et al.* 1991; Gill and Whitaker 1996; Crossland *et al.* 2005; Nájera-Hillman *et al.* 2009a), and has a non-feeding tadpole stage (Stephenson 1955b; Bell 1978a; Bell 1978b; Bell and Wassersug 2003). Assuming Hochstetter’s frog to be one species, it is classed as At Risk: Declining, but when considering the evolutionary significant units proposed by Fouquet *et al.* (2010), at least one population is classed as Threatened: Nationally Critical and 9 populations as At Risk: Declining (Newman *et al.* 2013).

Aside from native anuran fauna, New Zealand also has three species of introduced frogs, the southern bell frog (*Litoria raniformis*), the green and golden bell frog (*Litoria aurea*) and the brown (or whistling) tree frog (*Litoria ewingii*; Bishop 2008; Voros *et al.*, 2008). Southern bell frogs and green and golden bell frogs are listed as ‘endangered’ and ‘vulnerable’, respectively (IUCN 2013). Both are declining in their native ranges in Australia (IUCN 2013), where feral cats (*Felis catus*), ship rats (*Rattus rattus*), Norway rats (*R. norvegicus*) and house mice have been introduced. Although introduced fish, particularly mosquito fish (*Gambusia holbrooki*), are known to have a detrimental effect on bell frog populations (Pyke...
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and White 1997), very little research has been carried out on the impacts of terrestrial predators.

1.4 Evidence for the impact of introduced fauna on New Zealand’s native frogs

There is already evidence that introduced fauna have had an effect on New Zealand’s frogs. Here I discuss this evidence under three headings: Circumstantial evidence, Indirect evidence and Direct evidence. Circumstantial evidence is taken to be that based on present and past distributions of native frogs. Indirect evidence is that based on data collected from monitoring frog population changes or estimating the trophic position of frogs. Direct evidence pertains only to reports of frogs as prey, or reports of the interaction between frogs and potential predators. Although predation by introduced fauna is often referred to in the scientific literature as playing a major role in the decline of native New Zealand frogs, there have been no studies to date aiming to quantify direct evidence of predation on this taxon (Worthy 1987b; Bell 1994a; Bell and Bell 1994; Daugherty et al. 1994; Towns and Daugherty 1994; Holyoake et al. 2001; Bell et al. 2004a; Tocher and Pledger 2005; Baber et al. 2006; Haigh et al. 2007; Bell 2010; Bishop et al. 2013).

1.4.1 Circumstantial evidence of negative impacts of introduced fauna on native frogs

Fossil evidence indicates that prior to the arrival of humans Leiopelma ranged across both the North and South Islands of New Zealand (Worthy 1986; Worthy 1987b; Worthy 1987a; Worthy et al. 2013). The distributions of the four extant species have been reduced during human occupation of the country and their mainland habitats are still being modified today (Bell 1985; Towns and Daugherty 1994; Bishop et al. 2013). Their decline has been attributed to introduced fauna (Worthy 1987b; Towns and Daugherty 1994), habitat fragmentation (Waldman et al. 2001), disease (Bell et al. 2004a), pollution, and other poorly understood factors, such as the effects of climate change (Bishop et al. 2013). However,
agents of decline for native frogs have not been conclusively demonstrated (Bishop et al. 2013).

The evidence to date of introduced fauna negatively impacting Leiopelma, while convincing, is largely circumstantial. Most notable is that the extinction of three Leiopelma species occurred synchronously with the arrival of introduced mammals (i.e. the Pacific rat Rattus exulans, in association with human settlers) as did the range contraction of the currently extant species (Worthy 1987b; Bell 1994b; reviewed by Towns and Daugherty 1994). Furthermore, the only known naturally occurring populations of the Maud Island frog and Hamilton’s frog are restricted to mammal-free islands (although see note on recent mouse invasion on Maud Island in Section 1.3). Worthy (1987b) notes that the extinctions and range contractions are probably correlated with the arrival of the Pacific rat, and that the surprising thing is that Archey’s frog and Hochstetter’s frog still exist on the mainland at all.

### 1.4.2 Indirect evidence of predation on frogs in New Zealand

A number of studies in New Zealand have been carried out comparing frog abundance in areas where mammalian predators have been removed against those where no predator control was implemented (Mussett 2005; Baber et al. 2008; Nájera-Hillman et al. 2009b; Pledger 2011; Ohmer et al. 2012). The results to date have been varied. In the Waitakere Ranges, Nájera-Hillman et al. (2009b) found no difference in the relative abundance or size class of Hochstetter’s frogs between streams that had no rodent control and those that had very low rodent numbers (following 7 years of pest management). Conversely, Mussett (2005) and Baber et al. (2008) found that Hochstetter’s frog abundance was far higher in pest controlled areas than in non-pest controlled areas in the Hunua Ranges. However, the results of Mussett (2005) were complicated by the fact that in some pest control sites rat abundance was similar to that of non-pest control sites. Also, among pest control sites the highest rat abundance coincided with the highest frog abundance (Mussett 2005).
Between 2005 and 2011 the Department of Conservation (DOC) ran a predator removal experiment in Whareorino Forest whereby four 10 m x 10 m Archey’s frog population monitoring grids were set up, two in an area poisoned for rodents and two in a non-treatment area (Pledger 2011). Over that period frog population sizes remained stable or increased on grids inside the treatment area and decreased outside the treatment area (Pledger 2011). This supports the theory that rodents have a detrimental effect on Archey’s frog populations. However, the data were also incorporated into another model (Ohmer et al. 2012) that included predation (as per treatment block) and disease (frogs testing positive for chytridiomycosis) as factors. These authors did not find a functionally significant effect of predation and found that the population appears to be stable (Ohmer et al. 2012).

In the fenced sanctuary, Zealandia, a small population of Maud Island frogs (n = 58) were collected from Maud Island and divided into two even groups, with one group placed in a mouse-proof sub-enclosure and the second released in an area inhabited by mice (Lukis 2009). After one year, only one frog could be found outside the sub-enclosure, while 27 were recaptured inside the sub-enclosure (Lukis 2009). Since then, some more survivors and progeny have been found, but the small population has struggled to survive (BD Bell, unpublished data). However, other potential native predators (e.g. little spotted kiwi *Apteryx owenii* or morepork *Ninox novaeseelandiae*) are also present outside the sub-enclosure, so mice may not have been the cause of this. Another translocation took place in Zealandia in December 2012, with 100 Maud Island frogs being released into a fenced sub-enclosure that excludes kiwi, but not mice. That population is currently being monitored (BD Bell, unpublished data).

Finally, Najera-Hillman *et al.* (2009) investigated the trophic position of Hochstetter’s frog using stable isotope analysis and concluded that shortfin eels (*Anguilla australis*) and a fish species, banded kokopu (*Galaxias fasciatus*), might be more significant potential predators.
than ship rats. However, the results for the trophic position of ship rats in relation to frogs were inconclusive (Najera-Hillman et al. 2009).

### 1.4.3 Direct evidence of predation on frogs in New Zealand

To date there have only been a small number of isolated observations of direct evidence of predation on *Leiopelma*. A collation of all reported accounts of predation on both native and introduced frogs in New Zealand is provided in Table 1.1, and further details are given in Appendix A. It should be noted that this information does not represent the results of a systematic or restricted literature search *sensu* that of Toledo et al. (2007), rather it is a collation of all relevant reports found during literature searches pertaining to this topic.

One major difference between predation reports on frogs in New Zealand, compared to the global reports reviewed by Toledo et al. (2007), is the proportion attributed to introduced fauna (60% for New Zealand, 4% for global data; Figure 1.). This highlights the substantial impact that introduced fauna have had on the biodiversity in this country. On the other hand, given that Serpentes are not represented in New Zealand, the general trends appear to be quite similar, with Mammalia being the most reported, followed closely by Aves, then Anura, Actinopterygii and Reptilia (Figure 1.).

Interestingly, although weka (*Gallirallus australis*) are known to prey on introduced frogs (Table 1.1), in one experiment they quickly discarded native frogs (Hochstetter’s and Archey’s frogs) following an investigative tasting, suggesting that they were found to be unpalatable (Beauchamp 1996). Also, yelping by a Maud Island frog apparently deterred a New Zealand robin (*Petroica australis*) from continuing to peck at it and it proceeded to wipe its bill, perhaps removing distasteful skin secretions (Bell 1985). A banded kokopu and a tuatara (*Sphenodon punctatus*) also rejected *Leiopelma* (Bell 1982b; Bell 1985), however, both of these predators are known to consume *Leiopelma* species in the wild (Table 1.1). A ship rat was observed close to several immobile Archey’s frogs, but did not prey on them.
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(observed by C. Johnson and G. McBride in: Bell 1985). Such apparent distastefulness may be evidence of anti-predator defence. *Leiopelma* spp. have defensive skin glands (Green 1988; Melzer and Bishop 2010; Melzer et al. 2011; Melzer et al. 2012; Melzer et al. 2013) and laboratory Norway rats significantly preferred food covered with water over that covered with Maud Island frog skin secretions (Melzer et al. 2012). The Maud Island frog skin secretions also lysed rat red blood cells, but ingestion had no apparent ill effects on the rats (Melzer et al. 2012).

Figure 1.1 Percentage of the main vertebrate groups reported as predators of adult anurans and their status as native (■) or introduced (●). Global data (n = 243) is from Toledo et al. (2007), New Zealand data is from Table 1.1 (n = 20). *Note that the group Serpentes is not represented in New Zealand.
Table 1.1 Collation of all reported observations of predation events with either native or introduced frogs as prey in New Zealand. For species names, references and further details see Appendix A.

<table>
<thead>
<tr>
<th>Prey species</th>
<th>Predator Species</th>
<th>No. of predation events reported</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Leiopelma</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Archey’s frog</td>
<td>ship rat</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>green and golden bell frog</td>
<td>1</td>
</tr>
<tr>
<td>Hochstetter’s frog</td>
<td>ship rat</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>stoat</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>banded kokopu</td>
<td>1</td>
</tr>
<tr>
<td>Hamilton’s frog</td>
<td>tuatara</td>
<td>1</td>
</tr>
<tr>
<td>Hamilton’s frog or Maud Island frog†</td>
<td>laughing owl, extinct</td>
<td>8</td>
</tr>
<tr>
<td><strong>Litoria</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>green and golden bell frog</td>
<td>brown kiwi</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>weka</td>
<td>4+</td>
</tr>
<tr>
<td></td>
<td>Australasian harrier</td>
<td>6+</td>
</tr>
<tr>
<td></td>
<td>feral pig</td>
<td>1</td>
</tr>
<tr>
<td>brown tree frog</td>
<td>cat</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>ferret</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>southern bell frog</td>
<td>1+</td>
</tr>
<tr>
<td>southern bell frog</td>
<td>European hedgehog</td>
<td>10+</td>
</tr>
<tr>
<td></td>
<td>cat</td>
<td>2</td>
</tr>
<tr>
<td>southern bell frog or brown tree frog*</td>
<td>ferret</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>white-faced heron</td>
<td>2+</td>
</tr>
<tr>
<td></td>
<td>little owl</td>
<td>8</td>
</tr>
<tr>
<td>southern bell frog or green and golden bell frog†</td>
<td>little shag</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>† Not distinguished as separate species at the time of the study. * Not identified to species level.</td>
<td></td>
</tr>
</tbody>
</table>

1.5 Inferring the impact of predators on amphibians

One of the primary factors in community ecology dynamics is the process of predation (Leslie and Gower 1960; Murray 2002; Matia and Alam 2013). The term has been used in various ecological contexts to apply to processes such as parasitism, parasitoidism, herbivory and cannibalism (Elewa 2007). However, this thesis is concerned only with faunal predation sensu stricto, the killing and ingesting of one faunal organism by another.
Based on all known reports, it seems that introduced small mammal species may represent the greatest threat to New Zealand’s frogs, particularly rodents (Figure 1.1; Table 1.1).

Furthermore, ship rats, Norway rats and Pacific rats have reached about 90% of the world’s islands and are likely to pose a major threat to native amphibians in many countries (Innes 2005; Towns et al. 2006).

Various techniques to study predation are available. As mentioned in Section 1.4.2, there have already been indirect studies comparing frog abundances in the presence and absence of introduced predators. A major difficulty with comparing frog abundance estimates (e.g. at sites with and without mammal control) is that a difference in abundances may not reflect a difference in population size, but only in detection probability (Buckland et al. 2000; Yoccoz et al. 2001; Crossland et al. 2005), which can vary spatially or temporally (Anderson 2001; Hyde and Simons 2001; Anderson 2003; Bailey et al. 2004; Crossland et al. 2005). For example, Hochstetter’s frogs are found both in portions of streams with a high percentage cover of ground riparian vegetation and portions with very little ground vegetation (pers. obs.). Finding frogs is much more difficult where vegetation cover is higher, so attempting to directly compare the abundances of frogs between the two streams would not be effective without an estimate of detection probability. Furthermore, none of the studies so far have included a ‘before and after’ approach i.e. where monitoring commenced prior to predator removal and was continued for some time after. This approach would be likely to provide stronger evidence (Pledger 2011) and any trends would be more easily interpreted, as site differences (e.g. presence of disease or frog detectability) would not be expected to influence results as much. For example (Vredenburg 2004) observed that removal of introduced trout (Oncorhynchus mykiss and Salvelinus fontinalis) resulted in rapid recovery of frog (Rana muscosa) populations at multiple sites, compared with multiple control sites. The difficulty with this approach for New Zealand’s native frogs is that they are long-lived (Leiopelma pakeka reaching 37+ years; Bell and Pledger, 2010) and produce few eggs (1 - 22 eggs; Bell
and Wassersug 2003) relative to many other amphibians, so the before and after approach would necessitate a very long-term monitoring plan to detect population changes.

Stable isotope analysis is very useful for estimating the trophic position of an animal or detecting a temporal variation in predator diet (Najera-Hillman et al. 2009; van der Bank et al. 2011), however, it often fails to provide specific trophic interactions because isotopic values in prey can overlap (Carreon-Martínez and Heath 2010; Hardy et al. 2010). This approach also requires a complete knowledge of prey isotopic signatures, and these can be difficult to obtain (Corse et al. 2010), particularly for generalist predators. Using fatty acid analysis, prey forming part of a predator’s diet can sometimes be determined to species level, however, it incorporates a high level of uncertainty that necessitates complicated calibrations and this can sometimes produce inaccurate results (Williams and Buck 2010; Bowles et al. 2011).

Identifying the predator species responsible for the death of an animal is often possible through inspection of prey remains found in the wild (Sherrod et al. 1975; Haynes 1982; Jędrzejewski et al. 1992; Hart et al. 1996; Ratz et al. 1999; Lyver 2000; Cuthbert 2003; Draud et al. 2004; Selva et al. 2005; Whitworth et al. 2005; Stiner et al. 2012). This has been used to infer the predator species responsible for the death of frogs (Olson 1989; Woodward and Mitchell 1990; Bradford 1991; Thurley and Bell 1994b; Fitzgerald and Campbell 2002).

In some cases, predators exhibit very consistent feeding behaviour when ingesting frogs partially. For example, Beckmann and Shine (2011) observed that in every case of a raptor ingesting a cane toad (Rhinella marina), raptors had broken and peeled back the lower jaw, and removed the toad’s tongue. Bradford (1991) noted that Brewer's blackbirds (Euphagus cyanocephalus) commonly left behind the skeleton and rear limb skin of mountain yellow-legged frogs (Rana muscosa). As noted by Olson (1989) and Woodward and Mitchell (1990), where predators consume entire frogs, rather than partially ingest prey components, the potential for forensic pathology is removed.
1.5.1 Diet analysis

Analysis of diet, based on gastrointestinal tract or faecal contents is standard practice in ecology (Hyslop 1980; Corse et al. 2010), however, the successful identification of prey depends on an array of factors, including prey size and durability of identifiable parts (Major 1990), level of digestion prey that has been subject to prior to examination (Veron 1969), part of the prey ingested (Day 1966) and the degree of mastication effected by the predator (Hansson 1970; Kasper et al. 2004). Diet analysis can be undertaken using traditional morphological techniques, enzyme electrophoresis, immunological assays and, more recently, DNA-based methods (all reviewed by Symondson 2002).

The tendency of morphological diet analysis to underestimate the importance of prey, when only soft tissue has been ingested, or where prey are entirely soft-bodied, has often been noted (Goss-Custard and Jones 1976; Floyd and Jenssen 1983; Dickman and Huang 1988; Reeve 1994; González-Solís et al. 1997; Vincent et al. 1998; Gunzburger 1999; Tsipoura and Burger 1999; Sutela and Huusko 2000; Deagle et al. 2005b; Anderson et al. 2008; Byers et al. 2010; Brown et al. 2012; Boyer et al. 2013).

Amphibians generally do not possess hard forms of keratin (e.g. α-keratin or β-keratin) that comprise mammalian wool, hairs, quills, hooves, horns, nails and baleen, and bird feathers, bird scales and reptile scales (Marshall et al. 1991; Alibardi 2003). Keratin is highly resistant to digestion (Creer et al. 2002) and is frequently used for identifying prey in diet analyses (Gamberg and Atkinson 1988; Coyle et al. 1996; Iversen et al. 2013). So, unlike all other terrestrial vertebrates, the only resistant parts of an amphibian likely to survive mastication and digestion are the skeletal components, drastically reducing the diversity of material that can aid in prey identification.

Although post-metamorphic amphibians are consumed by a large array of vertebrate groups (reviewed by Toledo et al. 2007), many previous studies have identified difficulties in
identifying amphibians as prey in predator diets, particularly when attempting to obtain a fine
taxonomic resolution i.e. identifying amphibian components beyond class or order level (e.g.
Coman 1973; Roser and Lavers 1976; Chanin and Linn 1980; Webb *et al.* 1982; Nilsson
1984; Weber 1990; Harna 1993; King 1993; Martin *et al.* 1996; Barratt 1997; Ruiz-Olmo and
Palazón 1997; de Queiroz *et al.* 2001; Reif *et al.* 2001; de Aguiar and Di-Bernardo 2004;
Hammershøj *et al.* 2004; Clavero *et al.* 2005; Britton *et al.* 2006; Żmihorsk and Osojca 2006;
Kutt 2011).

Enzyme electrophoresis has been used in the past to compare enzyme banding patterns of
predator gut contents to those of prey (reviewed in Symondson 2002). The major drawbacks
are either a paucity of species-specific bands or the inability to separate complex banding
patterns when samples contain several prey species. Immunological assays involve the use of
antibodies, carefully selected to bind with a protein from a target prey species and these can
offer the detection of prey at almost any taxonomic level, or even a life stage within a species
(Symondson 2002). However, they can demand extensive resources, taking up to a year to
develop and requiring specialized tissue culture facilities.

DNA-based analysis of predation, i.e. polymerase chain reaction (PCR) amplification of prey
DNA within the faeces or digestive systems of predators, is a rapidly growing field (King *et
al.* 2008) that is used to study complex trophic interactions in the field and allows wildlife
managers to detect the presence of endangered taxa and manage their conservation (Farrell *et
al.* 2000). There have been very few studies comparing morphological and DNA-based diet
analyses, with the first being carried out by Casper *et al.* (2007b). However, studies that have
done so, have usually found that DNA-based methods improve prey detection success, either
by detecting prey more frequently, or by detecting a higher number of prey species (Scribner
and Bowman 1998; Purcell *et al.* 2004; Casper *et al.* 2007a; Casper *et al.* 2007b; Soininen *et
Casper et al. (2007b) found that detection for single target prey species increased by 37% for squid (Nototodarus sp.) and 21 - 22% for fish (Sillago spp. and Arripis georgianus), when analysing seal faeces (Arctocephalus spp.). Soininen et al. (2009) identified 75% of plant species to at least genus level using DNA-based methods, but only 20% when using microhistological methods in vole (Microtus oeconomus and Myodes rufocanus) stomach samples. Dunn et al. (2010) found that using DNA-based methods almost doubled the prey species richness for a range of shark species (Dalatias licha, Centrophorus squamosus and Centroscymnus owstoni). However, Casper et al. (2007a) found that using either morphological or DNA-methods underestimated the number of prey species in Antarctic fur seal (Arctocephalus gazella) faeces, and recommended combing both methods to achieve best results.

One of the reasons that there are relatively few studies comparing morphological and DNA-based methods for prey detection is that in many cases morphological analyses are simply not possible. For example, most invertebrates as predators are fluid feeders that consume no identifiable prey components, precluding morphological analysis (Sunderland 1988; Symondson 2002; Admassu et al. 2006; Greenstone et al. 2007; Pompanon et al. 2012). Some vertebrates, for example bats, are also known to heavily masticate their prey, leaving very few identifiable fragments, and researchers have exalted DNA-based methods as the only means to assess the range and diversity of bat diets (Clare et al. 2009; Zeale et al. 2010; Bohmann et al. 2011).

DNA-based prey identification techniques have often been applied to diet samples collected in vertebrate field studies (Höss et al. 1992; Taberlet and Fumagalli 1996; Reed et al. 1997; Scribner and Bowman 1998; Farrell et al. 2000; Nelson et al. 2000; Jarman et al. 2002;
Symondson 2002 [review]; Jarman et al. 2004; Jarman and Wilson 2004; Lee et al. 2006; Bradley et al. 2007; Deagle et al. 2007; Clare et al. 2009; Meekan et al. 2009; Soininen et al. 2009; Tollit et al. 2009; Corse et al. 2010; Dunn et al. 2010; Riemann et al. 2010; Zeale et al. 2010 and others; Rayé et al. 2011). However, it is the field of invertebrate ecology that has largely pioneered research in this area of molecular ecology (reviewed in Symondson 2002; Sheppard and Harwood 2005; and King et al. 2008). Many of these invertebrate studies have involved controlled laboratory experiments and feeding trials that have estimated prey detectability over time (since prey was ingested).

This has rarely been estimated for vertebrates and has never been studied in small mammals (Rosel and Kocher 2002; Deagle et al. 2005b; Casper et al. 2007b; Carreon-Martinez et al. 2011; Oehm et al. 2011; Hunter et al. 2012). Indeed, prey DNA detection rates have never been measured over time in any mammal stomach contents and prey DNA detection rates have never been compared, between stomach and faecal contents, over time for any vertebrate species.

Perhaps surprisingly, given the number of studies that have reported difficulties in identifying amphibians as prey (even beyond class or order level), there are no previous studies that have attempted to quantify the detection rate of amphibian prey in predator diet samples. In the context of introduced fauna impacting on endangered species, this is very important information to have. Many studies focusing on the impacts of introduced fauna rely on diet analysis (Park 2004). The number of prey consumed per unit time or area (predation rate) is a product of the density of the predator species and individual predators’ diets (Dempster 1960; Jones and Toft 2006). Therefore, in order to accurately estimate the predation rate, diet analysis can be used, but it is essential to account for the length of time that prey can be detected following predation, as this will have a direct effect on the number of field-collected samples testing positive for prey.
1.6 Thesis objectives and structure

The overall aim of this study was to aid global amphibian conservation by assessing, developing and utilising the tools available to determine the presence and extent of frog predation by introduced mammals. This is done through an assessment of predator feeding behaviour, an evaluation of traditional predator diet analysis and the development of novel DNA-based diet analysis, focusing on frogs as prey of introduced small mammals. Using New Zealand as a case study, I utilise novel diet analysis techniques to assess the impact of ship rats on rare and endangered frog species. The studies presented in individual chapters of this thesis address current knowledge gaps in the literature and are as follows:

Chapter 2 – The feeding behaviour of four introduced mammalian species presented frogs (*Litoria raniformis*) as prey

To assess whether small mammals will readily consume frogs or whether motivation was required, I presented frogs to four small mammal species (all previously implicated as being frog predators) under laboratory conditions. The aim of this research was to determine which mammal species are more likely to prey on frogs and under what conditions, thus helping wildlife managers to make more informed decisions on predator control operations to aid amphibian conservation. By collecting the remains of partially ingested frogs following laboratory feeding trials, I aimed to find out whether frogs found dead during field studies can reliably inform conservation researchers on the species identity of the small mammal predator responsible. I used southern bell frogs as a model prey species for this study, but aimed to draw conclusions that were applicable to a wide range of amphibians.
Chapter 3 - Detecting and identifying frogs as prey in the faecal and stomach contents of four small mammal species using morphological characteristics

In many diet studies prey items in predators’ digestive tracts or faeces can be reliably identified. However, this is not always the case and many researchers have noted that it can be very difficult to identify frogs as prey beyond class or order level. In this chapter, I highlight the difficulties involved in identifying frogs as prey of small mammals through traditional diet analysis methods. By measuring ingestion rate and time since prey ingestion I also aimed to disentangle the effects of ingestion and digestion on prey bone length, and to assess whether prey bone length in turn has an effect on the successful identification of frogs as prey. I use widely available methods in an effort to make the results meaningful to conservationists who may not necessarily have extensive experience in identifying frog bones.

Chapter 4 - DNA-based diet analysis to detect frogs as prey in small mammal stomach and faecal samples

In this study, I build on results from the previous chapters through the development of DNA-based protocols for detecting frogs as prey in predator stomach and faecal samples. I compare the efficacy of DNA-based methods against morphological methods. I aimed to provide the first reported measurement of prey detectability over time in mammal stomach contents, and the first comparison of DNA-based prey detection over time in stomach and faecal contents of any vertebrate. I provide a quantification of prey detection rates for frogs in small mammal diets, which can be used in predation rate estimating models. I provide valid ecological tools for detecting frogs as prey in predator stomach and faecal samples and provide further information on detecting the prey of small mammals in general, which will aid both herpetologists and mammalogists using diet analysis.
Chapter 5 - Estimating the predation impact of small mammals on three frog species in New Zealand

Research into the effects of introduced fauna on native New Zealand frogs, including the measurement of direct predation rates, has been termed “lacking”, “needed”, “a priority” and “urgent” by a number of researchers (Bell 1982b; Bell 1985; Bell 1994a; Baber et al. 2008; Najera-Hillman et al. 2009; Bell 2010). Action 14.1 of the current native frog recovery plan (Bishop et al. 2013) aims to “Determine the impacts of rats, mice and pigs on native frog populations by 2017.” Using the information obtained and tools developed in Chapter 4, I surveyed for predation by small mammals on three frog species in New Zealand through kill-trapping, faecal collection and subsequent diet analysis. I incorporated prey detection rates from the previous chapter to estimate the rate of predation by ship rats on endangered native New Zealand frogs. This provides a validation of the diet analysis techniques investigated in earlier chapters, and provided a case study that can be followed by future conservationists researching the impacts of introduced fauna on native amphibians.

Chapter 6 – General discussion

This chapter reviews the major outcomes and implications of the research presented in this thesis in the context of global amphibian declines, diet analysis in general, and the current and future role of diet analysis in conservation. It also conveys how the results fit with current understanding of these topics, outlines the knowledge gaps that this thesis has helped to fill and provides recommendations for wildlife management and future research.

1.7 Contributions

Although the majority of the work detailed in this thesis was undertaken by me, many people have contributed and without them this research would not have been possible. My supervisors, Associate Professor Phillip Bishop and Dr. Bruce Robertson, provided guidance
at all stages, including project planning, experimental design, interpretation and draft revision. My primary supervisor, Phil, also provided help with funding applications, animal ethics permits, field-collection permits, study site selection and tissue sample acquisition. My co-supervisor, Bruce, provided training in PCR methodology and concepts, as well as providing detailed advice on primer design and PCR optimisation steps.

Kim Garrett provided training for the use of animals in laboratory feeding trials and field trapping. Dr. Yolanda van Heezik provided advice on many occasions on the placement and use of live traps to obtain animals for feeding trial experiments. Dr. Tania King and Nicky McHugh both provided training for the range of equipment necessary to carry out analyses for the project. Ken Miller and Murray McKenzie built the infra-red lamps used in feeding trials and offered advice on the video recording set up to achieve best results. Cailín Roe helped to process the many samples assayed for the presence of frogs in Chapters 4 and 5. Manna Warburton and Luke Easton both provided advice on the statistics used in Chapter 3. Finally, without the help of the large number of volunteer field workers mentioned in the Acknowledgements, the predator stomach and faecal samples collected for Chapter 5 would not have been possible.
Chapter 2  The feeding behaviour of four introduced small mammal species presented frogs (*Litoria raniformis*) as prey

Abstract

The spread of introduced fauna has become accepted as a major factor in the decline of amphibians worldwide. Determining the impact of introduced predators is vital if management to protect endangered amphibians is to be implemented. The motivation required for predators to ingest certain prey under laboratory conditions can indicate which species are more likely to be predators under natural conditions. Furthermore, through forensic pathology, prey remains can be used to determine the predator species responsible for the death of an animal. The aim of this study was to examine the feeding behaviour of wild ship rats (*Rattus rattus*), mice (*Mus musculus*), hedgehogs (*Erinaceus europaeus*) and laboratory Norway rats (*Rattus norvegicus*) when presented southern bell frogs (*Litoria raniformis*) and to determine whether forensic pathology can be a useful tool for amphibian conservation. Hedgehogs and laboratory Norway rats showed a distinct preference to ingest (entire) southern bell frogs, even when other food was available (precluding forensic pathology). Ship rats and mice required motivation to (partially) ingest frogs. No frog body parts were ingested preferentially over any others, although ship rats tended to avoid ingesting rear feet and gastrointestinal tracts and tended to ingest the head. A low proportion of frog carcasses showed bite marks evident, and where present they ranged widely in length, likely due to the delicate nature of frog skin. This study highlights that caution is required when using forensic pathology to determine the predator species responsible for the death of a frog.
2.1 Introduction

The spread of introduced fauna has become accepted as a major factor in the decline of amphibians worldwide (Kats and Ferrer 2003). Analysis of diet based on stomach contents is standard practice in ecology (Hyslop 1980; Corse et al. 2010) and is often used to determine the impacts of introduced predators on native fauna. However, many previous studies have identified difficulties in identifying amphibian remains in predator stomach or faecal samples (e.g. Coman 1973; Roser and Lavers 1976; Chanin and Linn 1980; Webb et al. 1982; Nilsson 1984; Weber 1990; Harna 1993; King 1993; Martin et al. 1996; Barratt 1997; Ruiz-Olmo and Palazón 1997; de Queiroz et al. 2001; Reif et al. 2001; de Aguiar and Di-Bernardo 2004; Hammershøj et al. 2004; Clavero et al. 2005; Britton et al. 2006; Žmihorsk and Osojca 2006; Kutt 2011). It may be possible to obtain more information concerning predation on frogs, by complementing diet studies with additional pertinent information regarding predator feeding behaviours, and their physical effects on prey.

2.1.1 Feeding behaviour and prey selection

Introduced rodents are often thought of as being the ultimate generalists and opportunists (Emlen 1973; Le Roux et al. 2002). However, Clark (1981; 1982) showed that while ship rats (Rattus rattus) do eat a broad range of foods, they are also highly selective and do not simply feed indiscriminately. Prey selection in the natural environment will depend on a number of factors, including preference for certain prey (Rodgers 1990). The presentation of various prey in equal amounts to potential predators under laboratory conditions (known as cafeteria trials) has often been undertaken to assess predators’ food preferences (e.g. Dickman 1988; Rodgers 1990; Bellocq and Smith 1994; Murray and Dickman 1994; Day et al. 1998 [review]; Fullerton et al. 1998; Lundkvist et al. 2003; Melzer et al. 2012). Prey preference under these conditions is independent of availability (Litvaitis 2000), however, if food availability is low, predators may be forced to utilise less favourable foods (Rodgers 1990).
So, the motivation required (e.g. period of low food availability) to ingest certain prey under laboratory conditions can indicate which predator species may be a more likely predator under natural conditions. Cafeteria trials can be affected by predators’ learned responses to prey (Pennell and Rolston 2003) or by differences in the level of neophobic response exhibited by different predators (Catanese et al. 2012), and previous exposure to various foods has been shown to reduce neophobic responses in rats (Barnett 1958; Sahakian et al. 1982), so these should be accounted for in such studies.

2.1.2 Forensic pathology of partially ingested prey

Forensic pathology of prey carcasses found in the wild can often be used to indicate the predator species responsible for causing the death of the prey (reviewed in Stroud 1998), however, it can also be extremely difficult to confirm in many instances (Connolly 1992). The Department of Conservation (DOC) and other researchers have attributed the death of a number of Archey’s frogs (Leiopelma archeyi), found at Whareorino Forest, to predation by ship rats, based on the lengths and shape of bite marks present on frog carcasses (Thurley and Bell 1994b; Fitzgerald and Campbell 2002). Small mammal bite marks found on prey carcasses have also been used in other studies to implicate the predator species responsible (e.g. Lyver 2000; Cuthbert 2003; Draud et al. 2004; Whitworth et al. 2005), but the success rate can vary depending on the prey species involved (Ratz et al. 1999; Lyver 2000). To ensure that predators are identified correctly, it is important to know how reliable bite marks can be in predator identification.

Further clues to determine the predator species responsible can come from carcasses found in the wild that were only partially consumed by the predator, as certain predators may have preferences for certain body parts (Sih 1980; Lucas 1985; Shure et al. 1989; Gende et al. 2001). This is often due to different body parts possessing different energetic or nutritional value (Chen et al. 2004) or because certain body parts are unpalatable or toxic (Lizana and
Pérez Mellado 1990; Williams et al. 2003). For example, some raptors are known to only consume the tongues of cane toads (*Rhinella marinus*) to avoid their highly toxic skin (Beckmann and Shine 2011). Amphibians often possess such noxious agents stored in granular skin glands (Kats et al. 1988; Bevins and Zasloff 1990 [review]; Daly 1995 [review]; Clarke 1997; Williams et al. 2000; Melzer and Bishop 2010; Melzer et al. 2012). These are frequently positioned on the dorsal side of frogs, and frogs are known to tilt their dorsum towards predators as a defence mechanism (Toledo and Jared 1995; Toledo et al. 2011). The presence of skin secretions has been noted in all three *Litoria* species present in New Zealand (Williams et al. 2000), and although descriptions of their skin glands were recently published (Melzer et al. in press-a), the role of their skin secretions as anti-predator defence mechanisms is not yet fully understood. However, the secretions of green and golden bell frogs (*Litoria aurea*), the sister species to southern bell frogs (*Litoria raniformis*), did induce death in several frog species after contact (Humphries 1979). Kleinpaste (1990) noted that kiwi (*Apteryx mantelli*) faeces containing green and golden bell frogs as prey were quite undigested and concluded that the ingestion of the frogs led to diarrhoea in kiwi, and that the low incidence of green and golden bell frogs in kiwi scats was evidence that they quickly learn to avoid these frogs as prey.

### 2.1.3 Aims

The overall aim of the research presented in this chapter was to investigate the feeding behaviour of four small mammal species (Norway rat *Rattus norvegicus*, ship rat, house mouse *Mus musculus* and European hedgehog *Erinaceus europaeus*) when presented with frogs as prey (southern bell frogs). Empirical evaluation of mammalian bite marks on prey carcasses, or predator preference for certain prey body parts, to determine the species of predator responsible for prey death, has not previously been carried out for frogs as prey. While it was not specifically an aim of this research to determine the defensive mechanisms
of southern bell frog skin secretions, experiments were carried out to ensure that any potential effects could be accounted for and these are discussed throughout.

It should be noted that native New Zealand frogs (*Leiopelma* spp.) were not used in feeding trials, due to their high conservation status (IUCN 2013). Southern bell frogs were introduced to New Zealand and as such are not considered a priority for conservation in this country, or afforded protection under the Wildlife Act (1953; see Bishop, 2008 for more information). This allowed for individuals to be collected from the natural environment without impacting on conservationally important species.

The small mammal species included in this study were chosen for the following reasons:

1. The southern bell frog is globally endangered and declining in its native Australian range (IUCN 2013), where ship rats and house mice have been introduced (Downes *et al.* 1997; Hocking and Driessen 2000). Ship rats are known to prey on green and golden bell frogs (*Litoria aurea*; Pyke and White, 2001).

2. The decline of New Zealand frog species (*Leiopelma* spp.) has often been attributed to the introduction of rodents (Towns and Daugherty 1994), and ship rats are known to prey on Archey’s frogs (*L. archeyi*; Thurley and Bell, 1994; Fitzgerald and Campbell, 2002).

3. Norway rats, ship rats and mice are commonly implicated as conservation problems (reviewed in Atkinson 1985 and; Innes 2005; Towns *et al.* 2006), having reached about 90 % of the world’s islands (Phillips *et al.* 2007), and may be detrimental to frog species in other countries.

4. Although hedgehogs are not found in Australia, they are known to prey on frogs (including southern bell frogs in New Zealand), sometimes comprising a large portion
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of the diet (Brockie 1959; Herter 1965; Burton 1969; Morris 1983; Reeve 1994; Stewart 2012). While the hedgehog is generally considered to be a lowland species common only in modified habitat, substantial hedgehog populations have also been reported in native forest, the favoured habitat of New Zealand native frogs, far from any roads or tracks (King et al. 1996). In addition, hedgehogs have been noted less than 700 m from native frog (L. hochstetteri) populations in the Waitakere Ranges, Auckland (pers. obs.).

The specific aims of the research presented in this chapter are as follows:

1. To investigate whether rodents or hedgehogs exhibit preferences for southern bell frogs as prey and, where necessary, to measure the motivation required to ingest southern bell frogs. This will include experiments designed to account for predator learned behaviour (i.e. neophobic responses) and the potential presence of anti-predator skin defences.

2. To measure any resultant rodent or hedgehogs bite marks on frog carcasses and examine the frequency with which bite marks occur, and to ascertain whether certain frog body parts are avoided, so as to provide a baseline for researchers assessing the cause of death of frogs found in the wild.

2.2 Methods

2.2.1 Animal sourcing

It should be noted that all Norway rats (Long-Evans strain; n = 41; all 5 to 8 months old) used in this study were sourced from the University of Otago Taieri Resource Unit and as such their behaviour and digestive traits may not reflect those of wild Norway rats. They were utilised here as a model small mammal species to allow larger sample sizes and additional experiments than would be have been possible had only wild Norway rats been
sought. Henceforth they are referred to as “Laboratory Norway rats” in order to distinguish this point.

Wild ship rats (n = 16) and mice (n = 18) were caught in live-capture folding aluminium box traps (33 cm x 9 cm x 10 cm; Elliott Scientific, Upwey, Victoria, Australia) baited with oats and peanut butter following Wilson et al. (2007). Wild hedgehogs (n = 28) were caught in similar, but larger, traps (15 cm x 15.5 cm x 46 cm; Elliott Scientific, Upwey, Victoria, Australia) baited with minced rabbit meat following Cameron et al. (2005). Additional food and bedding (synthetic batting) was provided to ensure adequate sustenance and warmth for trapped animals (Wilson et al. 2007). Animals were trapped in the vicinity of Dunedin from the following locations (latitude longitude co-ordinates use the World Geodetic System 1984 and are reported in decimal degrees): Okia Reserve, Otago Peninsula (-45.821861 170.719814); Woodhaugh Gardens, Dunedin (-45.855168 170.510173); The Borrow Pits, Momona (-45.971704 170.164318); Orokonui Ecosanctuary, Waitatai (-45.777566 170.602334); Silver stream, Mosgiel (-45.809568 170.421724). Traps were situated as near as possible to known introduced frog (southern bell frog and/or brown tree frog *Litoria ewingii*) breeding areas in order to increase the likelihood of trapping mammals that had previously encountered these species. Both male and female animals ranging from immature to mature were trapped and subsequently transported to the animal laboratory in the Department of Zoology.

Southern bell frogs (n = 154) were hand-caught using nets from four field locations: The Borrow Pits, Momona (-45.971704 170.164318); Sweet Koura Farm, Alexandra (-45.221196 169.370255); Corrigall’s Farm, Omakau (-45.107842 169.554857); Rangiora, Christchurch (-43.324179 172.588348). In order to draw comparisons to native New Zealand frog species, individuals were chosen that had a snout-vent length (SVL) of between 22 and 40 mm (mean SVL of actual frogs captured = 32 mm). This was based on the mean SVL of Archey’s frogs
Chapter 2 – Feeding behaviour of mammals presented frogs

and Hochstetter’s frogs reported by Thurley and Bell (1994b), Baber et al. (2006), Tessier et al. (1991) and Bell et al. (2004a).

2.2.2 Animal husbandry

All mammals were housed in a temperature controlled room with 10 hour light / dark cycles and 2 hour ramp up / ramp down periods. Temperatures were set to 23 °C during light hours and 20 °C during dark hours. Access to water and food remained *ad libitum* to all animals during the pre- and post-experiment periods. Wild-caught mammals were initially trialled on four different foodstuffs: rabbit mince, salmon-flavoured wet cat food, rabbit-flavoured wet cat food and dry chicken-flavoured cat food. The dry cat food was unanimously favoured, with very little or none of the other foods being consumed over two days (data not shown). From this point on wild-caught mammals were only fed dry cat food (Friskies, Purina, New Zealand). Laboratory Norway rats were fed dry rat and mouse pellets (Specialty Feeds Pty. Ltd., Glen Forrest, Australia). Laboratory Norway rats had been raised on these pellets and they meet the American National Research Council’s guidelines for rat nutritional requirements. The dry cat food is made from similar ingredients and includes many of the same added vitamins and minerals. Pine woodchip bedding and shredded paper were used to line the floor of the cages and provided nesting material. These were replaced every 4 days.

Southern bell frogs were kept in group terraria (4 - 10 per terrarium) and were fed an assortment of live insects (mealworms, crickets, locusts and waxmoth larvae) weekly. Terraria were sprayed daily with filtered water and cleaned when necessary.

All potential predators were monitored daily to ensure they remained healthy throughout the experiments and were given at least 10 days to acclimatise to the laboratory environment before being subjected to feeding trial experiments.
2.2.3 Response of small mammals to frog presentations

For each feeding experiment, one southern bell frog was euthanized using a CO$_2$ chamber and subsequently presented to one (individually housed) small mammal subject, which remained on *ad libitum* availability of the regular food source. All experiments were carried out during the dusk stage of the lighting cycle, reflecting the time of day that hedgehogs and rodents are known to commence foraging and exhibit increased activity (Kristoffersson 1964; Borbély and Neuhaus 1978). As well as observing small mammals from an adjacent room via a one-way mirror, all experiments were video recorded (Sony Handycam DCR-TRV120 E-PAL) with the aid of infra-red lamps. Each trial lasted up to 12 hours (h), after which frogs were removed if they had not been ingested.

Each animal was subjected to two identical feeding trials (Trial 1 and Trial 2) spaced 5 - 7 days apart, to examine whether small mammals exhibited learned behaviour towards southern bell frogs as prey, which may have arisen from unpalatability, toxicity, or a neophobic / neophilic response. As a further test of the potential effect of anti-predator skin defences, an additional set of trials was carried out using a separate group of laboratory Norway rats, presented 2 g of frog muscle only. Faecal pellets were collected from rats that had ingested frogs with skin (*n* = 6) and rats that had ingested frog muscle only (*n* = 9), at 12 h, 18 h, 24 h and 30 h post-ingestion. The number of pellets produced and the average pellet dry weight (g) was recorded.

In cases where small mammals did not ingest frogs during Trials 1 and 2, a complete food restriction was implemented prior to the next presentation. An initial food restriction was implemented for 1 h prior to frog presentation. Again, trials lasted up to 12 h, after which frogs were removed if they had not been ingested. Where subjects still had not ingested a frog, further food restrictions were implemented (18 h, 24 h, 36 h or 48 h) and subjects were
presented frogs at each interval. These were carried out to estimate the motivation required for small mammals to consume a southern bell frog.

### 2.2.4 Forensic pathology

Where small mammals partially ingested frogs, carcasses were removed (after 12 h) and inspected under a dissecting microscope (Olympus SZ61, with Olympus DP25 digital camera attachment, Olympus Corporation, Tokyo, Japan) at between 6.7 X and 45 X magnification. Bite mark length was measured using the scale from the microscope photographs. Based on initial results, frogs were theoretically divided into the following separate body parts: head, forelimbs, rear limbs, rear feet, external abdomen (including muscle, vertebrae, pelvic girdle, pectoral girdle), gastrointestinal tract (GIT), and viscera (internal organs excluding GIT).

Note was also made of cases where small mammals actively avoided skin, while still attempting to ingest frogs. However, this was a rather subjective measure because small scraps of skin were usually ingested, even in cases where frog skin appeared to be generally ‘avoided’. Frogs were all placed in cages with the dorsal side facing upwards and note was made of whether mammals chose to ingest dorsal or ventral sides.

### 2.2.5 Statistical analyses

All statistical analyses were carried out using SPSS Version 20.0 (IBM Corporation, IBM SPSS Statistics for Windows, New York, U.S.A.).

Chi-squared tests were used to assess whether subjects were more likely to choose frogs as prey items within the first and second trials (i.e. whilst being fed ad libitum). Here, the null hypothesis was that the same proportion of small mammals chose to eat frogs and cat food, as those that chose to eat only cat food. To compare the responses between the two trials, within predator species, a two-tailed McNemar test (McNemar 1955) for related samples using binomial distribution and Exact significance was employed, treating the first trial as “before” and the second trial as “after”. This assessed whether subject choices changed during the
second trial. To compare those responses between mammal species, Mann-Whitney U-tests using Exact significance were performed (Mann and Whitney 1947). Chi-squared tests were also used to assess subject preference for certain frog body parts, each body part scoring a 1 or a 0, based on whether it had been ingested or not.

Probit regression was used to estimate the probability of small mammals to commence ingestion of a southern bell frog, under motivation (length of time without access to alternative food). Probit regression is often used to estimate the median lethal dose of a compound (Williams 1986; Takeuchi and Endo 2012). In this case, the length of time without access to alternative food was treated as the dose and the median response was the time at which 50% of subjects would ingest a frog.

A repeated measures one-way analysis of covariance (RM-ANCOVA) was performed to compare both the number of pellets and the dry weight of pellets produced by laboratory Norway rats that had consumed either entire frogs (with frog skin) or frog muscle only, while accounting for multiple observation from each subject as a random factor.

2.3 Results

2.3.1 Response of small mammals to frog presentations

A significantly higher proportion of hedgehogs (79%; $\chi^2_{1, n = 24} = 8.17, p < 0.005$) and laboratory Norway rats (88%; $\chi^2_{1, n = 16} = 9.0, p < 0.005$) ingested southern bell frogs while other foods were available during the first trial than would be expected under the null hypothesis (Figure 2.1), and these proportions were not significantly different from each other ($n = 40, U = 172, p = 0.7$). Conversely, a significantly lower proportion of ship rats (25%; $\chi^2_{1, n = 16} = 4.0, p < 0.05$) and mice (13%; $\chi^2_{1, n = 15} = 8.07, p < 0.005$) chose to ingest frogs than would be expected (Figure 2.1). These proportions also did not differ significantly from each other ($n = 31, U = 106, p = 0.6$). This suggests that wild hedgehogs will readily eat
southern bell frogs, even when alternative foods high in nutritional value are available, while wild ship rats and mice on average prefer other available food items over southern bell frogs.

The preference for food items did not change significantly between trials ($p = 1.0$ for all species – 2-tailed McNemar tests with Exact significance; Figure 2.1), indicating that small mammals did not exhibit a learned response to southern bell frog ingestion in these trials.

![Figure 2.1 Percentage of subjects that ingested southern bell frogs during the first (■) and second (■) feeding trials. No significant difference was found between trials. Significant differences among species are indicated by the letters above the bars. LNR – laboratory Norway rat.](image)

The majority of laboratory Norway rats and hedgehogs ingested a frog, either wholly or partially, while other food was available. Therefore, no further experiments were carried out to assess the motivation required to ingest a frog for these species. However, many ship rats and mice did not ingest any frog material during either of the first two trials. The motivation (length of time without alternative food source) required for 50 % of ship rats to commence ingestion of a southern bell frog was estimated as 3.3 h (95 % Confidence Intervals [CI]: 0, 14.04; Pearson Goodness-of-Fit: $\chi^2_{4, n = 16} = 7.58, p > 0.1$), while for mice it was 33.4 h (95 % CIs: 24.4, 50.46; Pearson Goodness-of-Fit: $\chi^2_{4, n = 16} = 4.06, p > 0.3$);
Figure 2.2). Alternative food was denied for up to 48 h and the probability of ingesting a frog increased with time (Figure 2.2). By 24 h, all ship rats had ingested part of a frog, whereas even at 48 h only 56 % of mice had done so. These results indicate that while both ship rats and mice will ingest southern bell frogs given a lack of an alternative food source, ship rats will ingest them far more readily than mice, and many mice might not ingest southern bell frogs at all, even when the metabolic requirement for sustenance is very high.

In 62 % of cases mice did not ingest any bone material, but they did not significantly avoid it either ($\chi^2_{1, n=13} = 0.69, p > 0.4$), while ship rats ingested bone material more often than would be expected (88 %; $\chi^2_{1, n=34} = 19.88, p < 0.001$). Ship rats and mice were observed to actively avoid skin in some cases (24 % and 23 %, respectively), however, ship rats significantly chose not to avoid skin ($\chi^2_{1, n=34} = 9.53, p < 0.005$), while mice showed no preference ($\chi^2_{1, n=13} = 3.77, p = 0.5$). It should be noted that this was a relatively subjective category (as it required observer judgement). Ship rats ingested both ventral and dorsal sides of southern bell frogs significantly more often than they ingested just one of the two sides ($\chi^2_{1, n=38} = 3.79, p = 0.05$). However, where ship rats did choose just one side, they significantly chose the ventral side ($\chi^2_{1, n=14} = 4.58, p < 0.05$), indicating the ventral side may be more palatable. All mice chose to ingest both the ventral and dorsal sides together. Laboratory Norway rats and hedgehogs generally consumed entire frogs, so did not show preference for ventral or dorsal sides.

### 2.3.1.1 Faecal output

There was no significant difference in faecal output between laboratory Norway rats that consumed frog skin ($n = 6$) or frog muscle tissue ($n = 9$) either in number of pellets excreted ($F_1 = 0.0002, p > 0.9$), dry weight of faeces overall ($F_1 = 0.18, p > 0.6$), or average faecal pellet dry weight ($F_1 = 0.01, p > 0.9$). This indicates that frog skin secretions did not affect the rate of faecal passage in these trials.
Figure 2.2 Probability that a ship rat (n = 16) or mouse (n = 16) will commence ingestion of a southern bell frog, under motivation (time without access to other food). Solid lines indicate responses predicted by probit regressions, dotted lines indicate 95% confidence intervals. Actual data (●) shows cumulative (n = 16 at each time point) results from subjects presented frogs at 0, 1, 18, 24, 36 and 48 h (0 h is used as a control group).
2.3.2 *Frog carcasses following small mammal predation*

Laboratory Norway rats and hedgehogs were more likely to ingest an entire frog, while ship rats and mice were more likely to only partially ingest frogs (notwithstanding that these are small frogs with a mean SVL = 32 mm; Table 2.1). Hence, for hedgehogs and laboratory Norway rats, no preference for certain frog body parts was evident and no statistical tests could be carried out. None of the frog body parts were ingested in a significantly greater proportion than any of the others by either ship rats ($\chi^2_{6, n = 238} = 11.8, p > 0.5$; Table 2.2) or mice ($\chi^2_{6, n = 91} = 1.42, p > 0.9$; Table 2.2). However, ship rats ingested the head of frogs significantly more often than would be expected under the null hypothesis (i.e. in significantly more than 50% of trails) and significantly avoided ingesting the rear feet and GIT (Table 2.2). Figure 2.3 and Appendix B show examples of frog carcasses following small mammal predation in this study.

**Table 2.1** Number of potential predators that ingested an entire frog, rather than partially ingesting a frog, during a feeding trial. Degrees of freedom (df) = 1 in all cases.

<table>
<thead>
<tr>
<th>Species</th>
<th>n</th>
<th>n ingested entire frog</th>
<th>n expected</th>
<th>$p$</th>
<th>$\chi^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>laboratory Norway rats</td>
<td>23</td>
<td>17</td>
<td>11.5</td>
<td>0.02</td>
<td>5.26</td>
</tr>
<tr>
<td>hedgehogs</td>
<td>25</td>
<td>24</td>
<td>12.5</td>
<td>&lt; 0.001</td>
<td>21.16</td>
</tr>
<tr>
<td>ship rats</td>
<td>17</td>
<td>1</td>
<td>8.5</td>
<td>&lt; 0.001</td>
<td>13.24</td>
</tr>
<tr>
<td>mice</td>
<td>12</td>
<td>1</td>
<td>6.5</td>
<td>0.002</td>
<td>9.31</td>
</tr>
</tbody>
</table>

**Table 2.2** Proportion of ship rats and mice that ingested respective frog body parts. None of the body parts were ingested in a significantly greater proportion than any of the others, but asterisks indicate body parts ingested in significantly more or less than 50% of trials. Degrees of freedom (df) = 1 in all cases. GIT – gastrointestinal tract.

<table>
<thead>
<tr>
<th>Frog body part</th>
<th>Ship rat</th>
<th>$\chi^2$, $p$</th>
<th>Mouse</th>
<th>$\chi^2$, $p$</th>
</tr>
</thead>
<tbody>
<tr>
<td>head</td>
<td>71 %*</td>
<td>5.77, $p &lt; 0.02$</td>
<td>46 %</td>
<td>0.08, $p &gt; 0.7$</td>
</tr>
<tr>
<td>forelimbs</td>
<td>56 %</td>
<td>0.47, $p &gt; 0.4$</td>
<td>46 %</td>
<td>0.08, $p &gt; 0.7$</td>
</tr>
<tr>
<td>rear limbs</td>
<td>53 %</td>
<td>0.19, $p &gt; 0.7$</td>
<td>46 %</td>
<td>0.08, $p &gt; 0.7$</td>
</tr>
<tr>
<td>rear feet</td>
<td>21 %*</td>
<td>11.77, $p &lt; 0.001$</td>
<td>54 %</td>
<td>0.08, $p &gt; 0.7$</td>
</tr>
<tr>
<td>external abdomen</td>
<td>44 %</td>
<td>0.47, $p &gt; 0.4$</td>
<td>38 %</td>
<td>0.69, $p &gt; 0.4$</td>
</tr>
<tr>
<td>viscera (excluding GIT)</td>
<td>53 %</td>
<td>0.19, $p &gt; 0.7$</td>
<td>31 %</td>
<td>1.92, $p &gt; 0.1$</td>
</tr>
<tr>
<td>GIT</td>
<td>32 %*</td>
<td>4.24, $p &lt; 0.05$</td>
<td>31 %</td>
<td>1.92, $p &gt; 0.1$</td>
</tr>
</tbody>
</table>
Chapter 2 – Feeding behaviour of mammals presented frogs

A relatively small number of frog carcasses had small mammal bite marks evident. In the case of laboratory Norway rats and hedgehogs, this was because the majority of frogs were entirely ingested (only 1 frog carcass had bite marks evident for each species). Although mice did not generally consume entire frogs, only one frog carcass had bite marks evident even though 11 carcasses were examined, while 9 (56 %) frog carcasses had bite marks following ship rat feeding. This precluded statistical comparison between species, but the fact that so few bite marks were evident is in itself an important note.

Figure 2.4 and Appendix C show examples of the evident bite marks. Multiple bite marks were present on a number of frog carcasses. Mean length of bite marks (Figure 2.5) were as follows: ship rats (mean = 0.73 mm, n = 23, SD = 0.3, range = 0.34 – 1.43 mm); mice (mean = 0.24 mm, n 10, SD = 0.1, range 0.12 – 0.45 mm); laboratory Norway rats (mean = 1.19 mm, n = 3, SD = 0.4, range = 0.86 – 1.63 mm); hedgehogs (mean = 1.65 mm, n = 3, SD = 1.14, range = 0.76 – 2.93 mm). The range of bite mark lengths is quite large. In some cases this may have been the result of multiple overlapping bite marks, or bite marks coupled with pulling or tearing may have resulted in larger lacerations.
Figure 2.3 Examples of frog carcasses following ship rat (A & B) and mouse (C & D) feeding trials. A: Ventral view of head and pectoral region, mandible and associated soft tissue removed. B: Ventral view of skull and dorsal view of abdomen and rear limbs – example of bone avoidance – almost all muscle and viscera removed with all skeletal components remaining. C: Dorsal view of abdominal region and rear limb - viscera and some muscle removed. D: Ventral view of anterior abdominal region and head – majority head removed, forelimbs remain, abdomen heavily masticated. Scales vary and are included on each plate. A more comprehensive photographic index is provided in Appendix B.

Figure 2.4 Examples of ship rat (A) and mouse (B) bite marks on frog carcasses. A: Ventral abdomen. B: Ventrolateral abdomen. Scales vary and are included on each plate. A more comprehensive photographic index is provided in Appendix C.
2.4 Discussion

2.4.1 Implications for southern bell frogs

All the small mammals included in this study ingested southern bell frogs, either wholly or partially. The response varied between species, with hedgehogs and laboratory Norway rats showing a distinct preference to ingest (entire) southern bell frogs, even when other food was available, while ship rats and mice required motivation to (partially) ingest frogs. This indicates that wild hedgehogs, and potentially wild Norway rats, will prey on frogs even when alternative high energy food sources are available, while ship rats and mice may only consume southern bell frogs when other foods are scarce. The results partially concur with reports of southern bell frogs as prey in the literature: hedgehogs are known to prey on them,
in some areas frequently (Brockie 1959; Stewart 2012); ship rats are known to prey on the closely related green and golden bell frog (Pyke and White 2001), but to date there have been no reports of either mouse, ship rat or Norway rat predation on southern bell frogs. Indeed, there are no reports that mice prey on any frog species in the wild and, given that less than 60 % of mice would resort to ingesting southern bell frogs even after 48 h without alternative food, it may be that predation rates in the wild are indeed very low.

In December 2012, Dr. Stephen Chadwick (Massey University, New Zealand) observed a hedgehog consuming a southern bell frog in the Manawatu district of New Zealand (Stewart, 2012; Figure 2.6). Based on the results in the present study, hedgehogs are likely to prey on southern bell frogs during a high proportion of frog encounters.

![Figure 2.6 Hedgehog ingesting a southern bell frog in New Zealand, courtesy of Dr. Stephen Chadwick, Massey University, New Zealand.](image)

Caution must be used if extrapolating the results from laboratory Norway rats in the present study (c. 90 % choosing to ingest southern bell frogs while other food was available) to their wild counterparts. Karli (1956) noted that only 12 % of laboratory Norway rats are spontaneous mouse-killers, but 70 % of wild Norway rats are, highlighting a distinct behavioural difference. However, if the experiments of Karli (1956) hold true for other prey, then perhaps wild Norway rats are even more likely to prey on southern bell frogs when encountered. Using the same strain of laboratory Norway rats as was used in the present
study (Long-Evans), Desisto and Huston (1970) found that almost all subjects killed live frogs (*Rana pipiens*), and some killed up to 30 frogs in successive, closely spaced, trials. Dead frogs were used in this study, which removed any stimulus of movement, and may have resulted in a lower ‘attack rate’ by small mammals. Albert *et al.* (1982) found that laboratory Norway rats responded more to live mice than to dead ones, with the median response to a live mouse described as sniffing followed by lunging attack within 2 seconds and the median response to a dead mouse being no attack or nibbling within 5 minutes of presentation.

### 2.4.2 Potential effects of southern bell frog skin defences

None of the mammal species showed a learned response to the presence of southern bell frogs, either negative or positive, indicating that little or no ill effect was produced by the ingestion of southern bell frogs. Mice have been shown to be (and are often regarded as) neophilic (Crowcroft 1966; Barnett and Smart 1975; Barnett *et al.* 1978), so their tendency in the present study to avoid southern bell frogs is perhaps even more striking. However, Southern (1954) and Kronenberger and Medioni (1985) found unpredictable and neophobic behaviour in mice respectively, so it does not appear that neophilia is a general rule for mice. The ingestion of southern bell frog skin did not affect the faecal output of laboratory Norway rats, indicating that anti-predator skin defences did not cause diarrhoea in this species, unlike observations of diarrhoea in kiwi that had ingested southern bell frogs (Kleinpaste 1990).

Nevertheless, the palatability of southern bell frog skin may have deterred feeding by ship rats and mice in the present study, as they significantly avoided frogs when other food was available and only partially ingested frogs in the absence of other food. Melzer *et al.* (2012) found that food coated with Maud Island frog (*Leiopelma pakeka*) skin secretions was consumed less by laboratory Norway rats, than food coated with water, but no ill effects on the rats were observed (even though the secretions were shown to effectively lyse rat red blood cells). A relevant note is that subjects in the present study were presented frogs that
had been euthanized at least one hour beforehand. Phillips and Shine (2007) found that while some frog species may remain toxic for months after death, in others the toxicity decreases rapidly after the frog has died, with *Litoria dahlii* becoming non-toxic to snakes (*Acanthophis praelongus*) 20 minutes after death.

Many predators modify their exposure to prey toxins by ingesting only selected body parts of prey (Williams *et al.* 2003). European otters (*Lutra lutra*) will ingest only the internal organs or posterior portions of common toads (*Bufo bufo*) to avoid their toxin glands (Lizana and Pérez Mellado 1990), while carcasses of newts (*Notophthalmus* spp.) that contain toxins have been found similarly disembowelled by unknown predators (Shure *et al.* 1989). Raptors are known to only consume the tongues of cane toads (*Rhinella marinus*) to avoid their highly toxic skin (Beckmann and Shine 2011). Palatability of amphibians is known to be lower in adult stages, when toxic glands have been fully developed, than in larval or juvenile stages (Formanowicz Jr and Brodie Jr 1982; Pearl and Hayes 2002). The glands that produce noxious skin secretions are often positioned on the dorsal side of frogs, and frogs often tilt their dorsum towards predators as a defence mechanism (Toledo and Jared 1995; Toledo *et al.* 2011). Ship rats in the present study ingested ventral portions of frogs more often than dorsal portions. However, Melzer *et al.* (*in press-b*) found that southern bell frogs displayed an even distribution of granular glands on both ventral and dorsal surfaces, so it is unlikely that the presence of dorsal glands caused this. It is possible that the ventral side of frogs was ingested more often because rats were attempting to reach internal organs, which may be easier through the ventral abdominal body wall.

Overall, the results indicate that ingesting southern bell frogs did not cause any ill effects on small mammals, but that ship rats and mice may be deterred by the apparent unpalatability of southern bell frogs. With a lack of alternative foods, all ship rats will ingest southern bell frogs, while less than 60% of mice will.
2.4.3 **Forensic pathology to determine predator species**

Hedgehogs and laboratory Norway rats generally ingested whole frogs and did not leave any carcasses to be inspected. Therefore, in the wild, few frog carcasses would be expected to be found following predation events by hedgehogs, precluding them from any determination of the predator species responsible via inspection of frog carcasses. However, the same may not to extend to wild Norway rats (see below).

Although no one body part was chosen more than any other, ship rats ingested the head of southern bell frogs more frequently than expected, while they generally avoided the rear feet and GIT. GIT avoidance in predators is often documented (e.g. Day 1968; White 1973; Mech and Boitani 2003), possibly because it may contain fermenting, potentially harmful, material. It is possible that rats were preferentially selecting the head, and with it the brain, as an energy rich component of the frog, as bears will do for salmon (Gende et al. 2001). It is unclear why ship rats tended to avoid the rear feet of frogs, but it may be because there is very little muscle in this body region, and a relatively large proportion of skin and bones, perhaps making them relatively unpalatable. Mice did not choose one body part over any other and also did not consume any body parts more frequently than would be expected. Overall, it appears difficult to determine which predator is responsible for causing a frog’s death, based on frog carcass body parts alone.

Fitzgerald and Campbell (2002) inspected eight *Leiopelma* frog carcasses suspected of rat predation in New Zealand. They found that in all cases the body cavity had been opened; five had all viscera missing, two had viscera pulled out, but still attached to the frog and one did not have viscera removed (Fitzgerald and Campbell 2002). The feeding behaviour of opening the body cavity and ingesting the viscera occurred in a similar proportion of rats in the present study. There is also some evidence that wild Norway rats exhibit similar feeding behaviour, as frog (*Rana temporaria*) carcasses with viscera removed were noted by Dr. Chris
Smal (*pers. comm.*; Figure 2.7), that were found in close proximity to a confirmed Norway rat burrow and had Norway rat faecal pellets present nearby.

**Figure 2.7** Frog (*Rana temporaria*) carcasses following suspected wild Norway rat predation, courtesy of Dr. Chris Smal, Ireland. A) One frog with viscera, three limbs, and muscle from left rear limb removed. B) Four frog carcasses with only viscera and muscle removed.

Small mammal bite marks found on prey carcasses have been used to implicate the predator species responsible (e.g. Lyver 2000; Cuthbert 2003; Draud *et al.* 2004; Whitworth *et al.* 2005), but the success rate can vary depending on the prey species involved (Ratz *et al.* 1999; Lyver 2000). In the present study, where frogs were only partially ingested, relatively few had any small mammal bite marks evident. This meant that statistical analysis could not be performed on bite mark size. Nonetheless, the absence of bite marks shows that this may not always be a reliable method to infer the predator species responsible. The range in bite mark sizes in the present study was large, and this is likely due to fragile nature of frog skin, coupled with multiple overlapping bite marks and tearing of frog skin to result in large lacerations. Ratz *et al.* (1999) also noted that clustered, overlapping bite marks can cause difficulty in identifying the predator species responsible. Using plastic chew cards, Sweetapple and Nugent (2011) were able to distinguish between small mammal species based on bite mark length (2 mm for ship rats, 0.5 mm for mice and 1 - 2 mm for hedgehogs), but also noted that mice sometimes gnawed bait without leaving bite marks on the cards. Thurley and Bell (1994b) discovered five *Leiopelma* frog carcasses in Whareorino forest, some with
bite marks evident, and suggested that predation by rats (most likely ship rats) was the cause. The results of the present study indicate that this may be more difficult when inspecting bite marks on frog skin, and caution must be taken when attempting to confidently determine the predator responsible. It is possible that small mammals would leave more bite marks if presented with live frogs, through killing or attack behaviour, but due to the delicate nature of frog skin, this would still be likely to result in a large range in bite mark lengths.

2.4.4 Implications for identifying frogs as prey in predator diet analysis

Many previous studies have identified difficulties in identifying amphibian remains in predator stomach or faecal samples, as it is often only possible to get to class or order level (e.g. Coman 1973; Roser and Lavers 1976; Chanin and Linn 1980; Webb et al. 1982; Nilsson 1984; Weber 1990; Harna 1993; King 1993; Martin et al. 1996; Barratt 1997; Ruiz-Olmo and Palazón 1997; de Queiroz et al. 2001; Reif et al. 2001; de Aguiar and Di-Bernardo 2004; Hammershøj et al. 2004; Clavero et al. 2005; Britton et al. 2006; Žmihorsk and Osojca 2006; Kutt 2011). The observations in the present study, coupled with evidence from Thurley and Bell (1994b), Fitzgerald and Campbell (2002) and Dr. Chris Smal (pers. comm.), show that where rodents are depredating frogs, they often do not consume prey entirely. Sometimes, they avoid all skeletal structures and consume only soft tissue body parts. This highlights that caution is required with the use of morphological techniques to detect frogs as prey in rodent stomach or faecal contents, as it may lead to false negatives. For example, a diet study of ship rats in Hawaii concluded that no rats had consumed Coquí frogs (*Eleutherodactylus coqui*; Beard and Pitt, 2006). However, in one instance during fieldwork in the same study area, a ship rat was observed carrying a Coquí frog (Woolbright *et al.* 2006). While it may have been the case that none of the rats studied had consumed frogs during the study period, it is also a possibility that the rats were consuming viscera and muscle tissue and very little or no skeletal material, and that predation events went undetected.
In contrast to the results of the present study, Brockie (1959) observed that in hedgehog faecal samples, frogs as prey were represented only by scraps of skin, and concluded that hedgehogs were avoiding the head and skeleton of southern bell frogs. Only one frog in the present study was not entirely ingested by hedgehogs, and it may be that the effects of mastication and digestion (investigated in Chapter 3 of this thesis) were responsible for the lack of identifiable bones in Brockie’s study (1959).

2.4.5 Conclusions

The results of this study show that wild ship rats and mice, both introduced to the native range of southern bell frogs in Tasmania (Hocking and Driessen 2000) and mainland Australia (Downes et al. 1997), will consume southern bell frogs and may pose a threat to this species, which is listed as endangered (IUCN 2013). This has not been demonstrated previously. However, the threat may be greatest when alternative food sources are low. When other food sources are available a relatively low proportion of ship rats or mice will ingest southern bell frogs as prey. The most likely explanation for this is that they find southern bell frogs unpalatable. Given no alternative, 100 % of ship rats will consume frogs upon encounter. Hedgehogs and laboratory Norway rats generally ingest entire frogs, while ship rats and mice tend to only partially ingest frogs. Clues for determining the predator responsible for the death of frogs found in the wild may be inferred from remaining body parts or bite marks, but there is considerable variation and caution should be used. Morphological identification of frogs as prey in rodent diet analysis may be of limited use, as a substantial proportion of rodents do not consume diagnostically viable components, only soft tissue.
Abstract

Wildlife management decisions regarding the control of introduced predators to protect endangered species are often based on predation rates derived from diet studies of the predators concerned. Many studies have noted difficulties in identifying amphibian remains in predator diet samples, particularly when attempting to identify prey to genus or species level. The aim of this study was to assess traditional morphological diet analysis as a method of detecting frogs as prey in small mammal stomach contents. Frogs (*Litoria raniformis*) were presented to wild ship rats (*Rattus rattus*), mice (*Mus musculus*), hedgehogs (*Erinaceus europaeus*) and laboratory Norway rats (*Rattus norvegicus*). Stomach and faecal contents were collected at various time intervals following frog ingestion and assayed for the presence of frogs as prey using morphological analysis. Only 11% of samples could be identified as containing frogs (to order level) and only 0.9% could be identified as containing southern bell frogs specifically. Maximum prey bone length (in stomach and faecal contents) was a significant factor affecting the successful identification of frogs as prey. Morphological diet analysis is of limited use, where small mammals have ingested frogs, due to the difficulties in identifying frog bones diagnostic of certain genera or species, the effects of small mammal ingestion on frog bones and the fact that small mammals often do not ingest the bones of frogs.
3.1 Introduction

Wildlife management decisions regarding the control of introduced predators to protect endangered species are often based on predation rates derived from diet studies of the predators concerned (Park 2004). To gather this important information from diet studies, it is essential that techniques exist to reliably detect prey remains. The traditional technique used to quantify predator diets is the morphological identification of prey remains in gastrointestinal tracts (GIT; Badan 1986; Risbey et al. 1999; McDonald et al. 2000; Jones et al. 2005; Jolley et al. 2010), scats (Arnaud et al. 1993; Ragg 1998; Bull 2000; Bartoszewicz and Zalewski 2003; Malo et al. 2004) or, in the case of birds of prey, regurgitated material (Smal 1987; Korpimaki and Norrdahl 1989; Lewis et al. 2004). While diet studies are undoubtedly a powerful tool for information collection, the problem of collecting accurate diet data is widespread in field ecology (Redpath et al. 2001). The successful identification of prey remains depends on an array of factors including prey size and durability of identifiable parts (Major 1990), the level of digestion prey has been subject to prior to examination (Veron 1969), the part of the prey ingested (Day 1966) and the degree of mastication effected by the predator (Hansson 1970; Kasper et al. 2004).

The identification of prey in predator diet samples is possible for many prey species or taxonomic groups by using reference collections of potential food items (Trites and Joy 2005), as prey may pass through the digestive system relatively unaltered (Kelly and Garton 1997). For example, otoliths are often used to identify fish remains (reviewed in: Hyslop 1980; Pierce and Boyle 1991), cranial structures and other bones to identify mammalian remains (Buckley and Goldsmith 1975; Andrews and Nesbit Evans 1983; Díaz-Ruiz et al. 2013), feathers to identify bird remains (Linden and Wikman 1983; Barrett et al. 2007), exoskeletons to identify insect remains (Dickman and Huang 1988; Michalski et al. 2011), and various plant structures to identify plants (Dove and Mayes 1996; de Iongh et al. 2011).
Amphibians generally possess neither hard $\alpha$-keratin, comprising mammalian wool, hairs, quills, hooves, horns, nails and baleen (Marshall et al. 1991), nor hard $\beta$-keratin, comprising bird feathers, bird scales and reptile scales (Alibardi 2003), although a few exceptions to this rule are found in salamanders and two genera of anura (Maddin et al. 2009). Both of these materials are highly resistant to digestion (Creer et al. 2002) and are frequently used for identifying prey remains in diets (Gamberg and Atkinson 1988; Coyle et al. 1996; Iversen et al. 2013). So, unlike all other terrestrial vertebrates, the only hard parts of an amphibian likely to survive mastication and digestion are the skeletal components, which drastically reduces the diversity of material that can aid in prey identification.

While frogs have often been identified as prey in mammalian diets (e.g. Brockie 1959; Coman 1973; Roser and Lavers 1976; Thurley and Bell 1994b; Pyke and White 2001; Flux 2007; Toledo et al. 2007 [review]; Morgan et al. 2009; Clapperton et al. 2011), many studies have noted difficulties in identifying amphibian remains in predator diet samples, particularly when attempting to obtain a fine taxonomic resolution i.e. identifying prey to genus or species level (e.g. Coman 1973; Roser and Lavers 1976; Chanin and Linn 1980; Webb et al. 1982; Nilsson 1984; Weber 1990; Harna 1993; King 1993; Martin et al. 1996; Barratt 1997; Ruiz-Olmo and Palazón 1997; de Queiroz et al. 2001; Reif et al. 2001; de Aguiar and Di-Bernardo 2004; Hammershøj et al. 2004; Clavero et al. 2005; Britton et al. 2006; Žmihorsk and Osojca 2006; Kutt 2011). However, that fine taxonomic resolution may often be required, for example, in areas that are inhabited by multiple species of a particular order, family or genus, but where only some of those species are considered to be of high conservation concern. This is likely to be a common scenario in many areas of the world and an example is evident in New Zealand where the two mainland endangered native frogs, Archey’s frog (*Leiopelma archeyi*) and Hochstetter’s frog (*Leiopelma hochstetteri*), not only occur together, but also often sympatrically with other frogs of the introduced genus *Litoria* (Thurley and Bell 1994a).

Highlighting the perishability of amphibian prey components, are the studies by Daltry et al.
(1998) and Creer et al. (2002). They were able to justify the assumption that the presence of invertebrates in the stomach or faecal samples of snakes, were actually the result of snakes ingesting amphibians. This was because the snakes were unlikely to prey on invertebrates directly and it was more likely that amphibians had preyed on invertebrates and then they in turn were preyed on by snakes. In many cases amphibian components were not found at all, testament to both the perishability of amphibian tissue and the durability of chitin.

Perhaps surprisingly, given the number of studies reporting difficulties, there are no previous studies that have explicitly attempted to quantify the detection rate of amphibian prey in predator diet samples. Pinto Llona and Andrews (1999) did investigate the breakage and digestive effects on amphibian bones found in the diet samples of a range of predator species, including European polecats (*Mustela putorius*), European otters (*Lutra lutra*) and European badgers (*Meles meles*), but their data set did not include measurements of predator mastication or time since prey ingestion. The length of time prey is detectable is essential information if attempting to estimate predation rates, as it directly affects the number of samples that will test positive for prey.

There is an urgent need to quantify predation by introduced predators on amphibians at a global level (discussed in Chapter 1 of this thesis), as it is ranked as the third most important factor in global amphibian declines (Chanson et al. 2008). The main aim of the research presented in this chapter was to assess traditional morphological analysis as a method to detect frogs as prey in small mammal stomach and faecal samples.

The specific aims of this study were to investigate:

1. Whether the rate at which frog prey is ingested reduces the bone size of prey.
2. Whether prey bones are further reduced in length by the effects of digestion.
3. Whether prey bone length affects the success rate of frog prey identification.
4. The efficacy of traditional morphological analysis in identifying frog prey remains to order, genus or species levels in laboratory Norway rat (*Rattus norvegicus*), ship rat (*Rattus rattus*), mouse (*Mus musculus*) and hedgehog (*Erinaceus europaeus*) stomach and faecal contents.

### 3.2 Methods

Details of animal sourcing and husbandry are provided in Chapter 2 of this thesis. For each feeding experiment, either for stomach sample collection or faecal sample collection, one southern bell frog (*Litoria raniformis*) was euthanized via a CO$_2$ chamber and subsequently presented to one (individually housed) mammal subject. All experiments were carried out during the dusk stage of the lighting cycle, reflecting the time of day when hedgehogs and rodents are known to commence foraging and exhibit increased activity (Kristoffersson 1964; Borbély and Neuhaus 1978), as this can substantially affect digestion rates (Loder *et al.* 1998). All feeding experiments were video recorded (Sony Handycam DCR-TRV120 E-PAL) with the aid of infra-red lamps.

#### 3.2.1 Measuring ingestion rate

To measure the ingestion rates (g/min) of the mammal species in the present study, the weight (g) of each frog was recorded prior to feeding experiments and the length of time (min) each predator spent ingesting frog prey was measured by analysing feeding trial video recordings. The ingestion rate, chew cycle frequency (chew cycles/min) and chewing intensity (chew cycles/g) of mammals are all directly proportional to their body mass (Pilbeam and Gould 1974; Gould 1975; Druzinsky 1993; Shipley *et al.* 1994; Gerstner and Gerstein 2008). Consequently, the ingestion rate of mammals is strongly correlated with the quotient of chew cycle frequency and chewing intensity (Shipley *et al.* 1994). Therefore, ingestion rate of mammals provides a useful measurement of the potential effects of mastication on prey.
Small mammal body mass (g) was recorded and the relationship between body mass and ingestion rate for the individuals in the present study was also explored.

3.2.2 Collection of faeces

Once a predator had consumed an entire frog (in the case of hedgehogs and laboratory Norway rats) or partial frog (in the case of ship rats and mice) faecal pellets were collected at regular 6 hour (h) intervals. It was sometimes necessary to first implement food restriction prior to frog ingestion (see details in Chapter 2) and where this was the case, alternative food was returned to the animal ad libitum immediately after frog ingestion. It was important not to allow any contamination between faecal collections as the samples collected were also to be used downstream for molecular analysis. Cages for this experiment consisted of a raised wire mesh floor through which rodent faecal pellets could fall, landing in a collection tray.

Faeces collection trays were changed between each collection and all faeces were stored in 95% ethanol (following Deagle et al. 2005b) at a 4 (ethanol):1 (diet sample) ratio (following Wasser et al. 1997). This approach also minimised the stress caused to the subjects, as it precluded the need to directly handle the animals, which is known to affect GIT transit in rodents (Enck et al. 1989). Within faecal sampling intervals, all faeces from each subject were collected as one sample and stored in one collection container. Forceps for collecting faecal samples were rinsed in 95% ethanol and flamed between faecal collections to remove any residues.

The number of collection intervals was based on known GIT transit times for the respective species. Laboratory Norway rat (Wistar strain) mean GIT transit is 15 – 22 h (Sigleo et al. 1984; Enck et al. 1989) therefore faeces were collected from 12 – 30 h for this species (n individuals = 6, n faecal samples = 24). The known GIT transit for mice is c. 10 h (Thompson et al. 2010) and faeces were collected from 6 – 30 h (n individuals = 10, n faecal samples = 49). No GIT transit data were available for ship rats - faeces were collected from 6 – 30 h (n
individuals = 8, n faecal samples = 48). See Section 3.2.2.1 for details on hedgehog faecal collection times.

### 3.2.2.1 Hedgehog faecal collection

It was not possible to use a raised wire mesh floor for the hedgehog cages. This was due to the viscous consistency of the hedgehog faeces, which caused it to stick to the wire mesh and the underside of the subject. In order to combat this, hedgehogs were briefly immersed in a series of 4 water baths (at room temperature of 23 °C) between trials to remove any residual faeces before being transferred into a new cage. However, this lead to two issues with the experimental design: 1) washing may not be 100 % effective and the washed hedgehog may have been carrying residual faecal matter into the next cage and; 2) excessive handling may lead to differing GIT transit times. The first issue is of particular importance as the faeces being collected were also to be examined downstream for molecular analysis (see Chapter 4), and any contamination would affect results. However, visible faecal material was indeed washed off and did not interfere with methods detailed in this chapter. Troubleshooting the potential carry-over contamination of prey DNA is discussed further in Chapter 4 of this thesis.

To assess the effect of washing on GIT transit 40 small plastic pellets were mixed in with the dry pet food normally fed to the hedgehogs. Twelve hedgehogs were divided into 2 groups of 6. Both groups were fed the pet food with plastic pellets. One group was washed every 3 h and the other (control group) was only moved from one cage to another at each interval. Faeces were examined from both groups every 3 h until 100 % of plastic pellets were recovered. The number of plastic pellets at each 3 h interval was noted. Based on the results (Section 3.3.1), hedgehog faeces were collected from 6 - 18 h post prey ingestion (n subjects = 14, n faecal samples = 51). The discrepancy between the number of subjects and the number of faecal samples is because not all hedgehogs had defecated during every 6 h
interval. Furthermore, the faeces of 5 hedgehogs were collected up to 30 h post-ingestion to ensure a robust data set.

3.2.3 Collection of stomach contents

As for faecal sample collection, feeding trials were carried out whereby one southern bell frog was euthanized via a CO₂ chamber and subsequently presented to one (individually housed) mammal subject. Where a subject had previously ingested a frog for inclusion in faecal collection trials, an interval of at least 4 days between trials was provided. To obtain stomach contents, the time that a subject consumed a frog was noted and the subject was then euthanized using a CO₂ chamber at either 1 h, 3 h, 6 h, 9 h or 12 h after prey ingestion. The number of subjects within each group, and the number of time intervals within each species, was based on a running model that was updated with prey detection data following each set of feeding trials. This ensured the minimum number of animals required, while ensuring the data obtained was enough to for a robust data set. It is important to note that the running model was based on the downstream molecular prey detection rates detailed in Chapter 4 of this thesis, and not on visual prey identification rates detailed herein. This is due to the paucity of positive observations using the latter technique (see Section 3.3.2).

During field trapping conditions, kill-trapped animals would not be collected and dissected until the morning following the trap night and stomach samples collected would not be analysed until field work had been completed and samples could be transferred to a suitable laboratory. To reflect this, carcasses of subjects were stored at 20 - 23 °C for 8 h prior to dissection, at which point entire stomachs were removed and placed in 95 % ethanol (following Deagle et al. 2005b) at a 4 (ethanol):1 (diet sample) ratio (following Wasser et al. 1997), and stored for 6 months or longer. Removing the entire stomach rather than emptying stomach contents into sample containers at this stage limited the potential for contamination.
Subjects were dissected on disposable bench covers and dissection instruments were scrubbed with ethanol and flamed between each dissection.

### 3.2.4 Morphological analysis of prey remains

Faecal and stomach contents were examined and photographed under a dissecting microscope (Olympus SZ61, with Olympus DP25 digital camera attachment, Olympus Corporation, Tokyo, Japan) at between 6.7 X and 45 X magnification. Reference southern bell frog specimens were used to compare and identify prey remains.

To measure the effects of ingestion rate and digestion length, the response variable chosen was the maximum bone length (mm) of each frog: 1) prior to being subjected to ingestion and; 2) post ingestion (i.e. in predator stomach or faecal contents). The longest bone in a frog is the tibiofibula (Holmes 1906; Marshall 1930). Dissection of every frog, to measure the tibiofibula directly, could have affected feeding trials. To avoid this, the ratio of the snout-to-vent length (SVL):tibiofibula length was estimated by dissecting 11 southern bell frogs (mean = 0.42; range: 0.39 – 0.46 ± SD 0.02). Using this ratio, the tibiofibula length of each frog used in feeding trials was estimated from the SVL. Frog SVL is highly correlated with any measure of frog body size (Blouin and Loeb 1991) and tibiofibula length:SVL ratio does not vary greatly within species, particularly where frogs have a similar SVL (Martof and Humphries 1959; Emerson 1978; Emerson 1986). Post-ingestion (i.e. in a stomach or faecal sample), the maximum bone length was simply the length of the longest bone, or bone shard, that could be found in a sample. Measurements (mm) were made using the scale on the microscope and subsequent microscope photographs.

While there was an obvious *a priori* knowledge that subjects had consumed southern bell frogs, samples were treated as if they had come from an unknown New Zealand location and could potentially have contained any frog species present in New Zealand. This more general approach was used so as to make the results more interpretable from a global perspective.
where in different areas a limited number of known anuran species may be present and traits used to identify anuran prey components will be based on those species. The traits used in the current study to identify prey to order level (Table 3.1), genus level (Table 3.2), or species level (Table 3.3) are provided (see also Figure 3.1). As all frogs used in feeding trials were of a similar size to native New Zealand frog species, length of bones alone was not a useful predictor of prey species.

It is important to note that it was not possible to identify any frog remains in mouse stomach or faecal samples because they did not consume skeletal components in the trials preceding euthanasia or faecal sample collection (see Chapter 2 for details of frog body parts ingested by small mammals). Therefore, mice were excluded from all analyses, bar those pertaining only to ingestion rate.

Table 3.1 Traits used to identify prey components as belonging to the order anura.

<table>
<thead>
<tr>
<th>Prey component</th>
<th>Traits</th>
</tr>
</thead>
<tbody>
<tr>
<td>Astragalus and calcaneum</td>
<td>Greatly and obviously elongated compared to other vertebrates, fused at distal and proximal ends</td>
</tr>
<tr>
<td>Ilium</td>
<td>Greatly and obviously elongated compared to other vertebrates</td>
</tr>
<tr>
<td>Maxilla</td>
<td>Possessing row of pleurodont, homodont, similar sized, pedicellate, cone teeth</td>
</tr>
<tr>
<td>Mandible</td>
<td>Mentomeckelian bones present, non-dentate (^1)</td>
</tr>
<tr>
<td>Radioulna</td>
<td>Fusion of radius and ulna unique to anura</td>
</tr>
<tr>
<td>Tibiofibula</td>
<td>Fusion of tibia and fibula unique to anura</td>
</tr>
</tbody>
</table>

Adapted from Holmes (1906), Marshall (1930), Kotpal (2010) and Sharma (2010).

\(^1\) Owen (1840), Parsons and Williams (1963)
Chapter 3 – Detecting frogs as prey using morphological analysis

Figure 3.1 Diagram of the skeleton of a frog (Pelophylax esculentus). Labels include only those bones identified from small mammal stomach and faecal samples in the present study (detailed in this Chapter and Appendices D and E). Adapted from Boulenger (1890).
Table 3.2 Traits used to identify prey components as belonging either to genus *Litoria* or *Leiopelma*. Note that these traits were used only to identify components of potential mainland anuran species in New Zealand and traits listed do not necessarily extend to offshore or extinct species of either genus.

<table>
<thead>
<tr>
<th>Prey component</th>
<th>Trait - <em>Litoria</em></th>
<th>Trait - <em>Leiopelma</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Urostyle</td>
<td>Transverse processes absent(^1)</td>
<td>Transverse processes present(^2)</td>
</tr>
<tr>
<td>Vertebræ</td>
<td>Procoelous presacral vertebrae(^3)</td>
<td>Amphicoelous presacral vertebrae(^4)</td>
</tr>
<tr>
<td>Humerus</td>
<td>Proximal end curved medially, humeral crest present</td>
<td>Proximal end straight (<em>L. hochstetteri</em>), or lacking humeral crest (<em>L. archeyi</em>)(^2)</td>
</tr>
<tr>
<td>Sacral vertebra</td>
<td>Single bone(^5)</td>
<td>Two separate bones joined by cartilage(^5)</td>
</tr>
<tr>
<td>Radioulna</td>
<td>Fusion boundary evident along entire shaft</td>
<td>Fusion boundary not evident in mid-shaft region(^2)</td>
</tr>
<tr>
<td>Femur</td>
<td>Relatively less sigmoid in shape(^2)</td>
<td>Distinctly sigmoid(^2)</td>
</tr>
<tr>
<td></td>
<td>Relatively narrow at both ends(^2)</td>
<td>Relatively wider at ends(^2)</td>
</tr>
<tr>
<td></td>
<td>Relatively weak femoral crest(^2)</td>
<td>Relatively strong femoral crest(^2)</td>
</tr>
<tr>
<td>Ilium</td>
<td>Lateral groove present(^6,7)</td>
<td>Lateral groove absent(^2)</td>
</tr>
<tr>
<td>Digit terminal discs</td>
<td>Present(^8)</td>
<td>Absent(^9,10)</td>
</tr>
</tbody>
</table>

\(^1\) Holman (2003); \(^2\) Worthy (1987a); \(^3\) Tyler and Davies (1985); \(^4\) Stephenson (1952); \(^5\) Stephenson (1960); \(^6\) Tyler (1977); \(^7\) Tyler (1986); \(^8\) Courtice and Grigg (1975); \(^9\) Cree (1989); \(^10\) (Turbott 1942);
### Table 3.3 Traits used to identify prey components as belonging to species (within the genus *Litoria* or *Leiopelma*). Note that these traits were used only to identify components of potential mainland anuran species in New Zealand and traits listed do not necessarily extend to offshore or extinct species of either genus.

<table>
<thead>
<tr>
<th>Genus</th>
<th>Prey component</th>
<th>Trait</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Rear interdigital webbing</td>
<td>Fully webbed apart from 4&lt;sup&gt;th&lt;/sup&gt; toe&lt;sup&gt;1&lt;/sup&gt; (brown tree frog, <em>Litoria ewingii</em>)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>50% - 75% webbed&lt;sup&gt;1&lt;/sup&gt; (green and golden bell frog, <em>Litoria aurea</em>)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Near complete webbing&lt;sup&gt;1&lt;/sup&gt; (<em>L. raniformis</em>)</td>
</tr>
<tr>
<td><em>Litoria</em></td>
<td>Terminal discs</td>
<td>Terminal discs wider than digit&lt;sup&gt;3&lt;/sup&gt; (<em>L. ewingii</em>)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Terminal discs wider than digit&lt;sup&gt;1&lt;/sup&gt; (<em>L. aurea</em>)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Terminal disc widths equal to digit widths&lt;sup&gt;1&lt;/sup&gt; (<em>L. raniformis</em></td>
</tr>
<tr>
<td></td>
<td>Rear interdigital webbing</td>
<td>Present, 50% webbed&lt;sup&gt;2&lt;/sup&gt; (<em>L. hochstetteri</em>)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Absent&lt;sup&gt;2&lt;/sup&gt; (<em>L. archeyi</em>)</td>
</tr>
<tr>
<td><em>Leiopelma</em></td>
<td>Ilium</td>
<td>Dorsal prominence and protuberance present&lt;sup&gt;4&lt;/sup&gt; (<em>L. hochstetteri</em>)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Dorsal prominence and protuberance absent&lt;sup&gt;4&lt;/sup&gt; (<em>L. archeyi</em>)</td>
</tr>
<tr>
<td><em>Leiopelma</em></td>
<td>Humerus</td>
<td>Proximal end curved medially, with humeral crest&lt;sup&gt;4&lt;/sup&gt; (<em>L. archeyi</em>)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Proximal end straight, without crest&lt;sup&gt;4&lt;/sup&gt; (<em>L. hochstetteri</em>)</td>
</tr>
<tr>
<td><em>Leiopelma</em></td>
<td>Maxilla</td>
<td>Notched pars facialis&lt;sup&gt;4&lt;/sup&gt; (<em>L. hochstetteri</em>)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Unnotched pars facialis&lt;sup&gt;4&lt;/sup&gt; (<em>L. archeyi</em>)</td>
</tr>
</tbody>
</table>

<sup>1</sup>Courtice and Grigg (1975); <sup>2</sup>Turbott (1942); <sup>3</sup>Watson <i>et al.</i> (1971); <sup>4</sup>Worthy (1987a)

### 3.2.5 Statistical analyses

All statistical analyses were carried out using SPSS Version 20.0 (IBM Corporation, IBM SPSS Statistics for Windows, New York, U.S.A.), unless otherwise stated.

A repeated measures analysis of variance (RM-ANOVA) was used to investigate whether washing hedgehogs had an effect on the GIT transit (as measured by the number of plastic pellets in faecal samples at various time intervals post ingestion). Mauchly’s Test of Sphericity (Crowder and Hand 1990) was used to test variables’ homoscedasticity, and where variables were heteroscedastistic, the Greenhouse-Geisser correction was applied – this is a
conservative corrected probability (Scheiner and Gurevitch 2001) and other adjustments can occasionally fail to control Type I errors (Maxwell and Delaney 2004).

Probit regression was performed based on the proportion of successful detections of frogs as prey (to order level) and used to estimate the half-life of prey detection (hours at which probability of detecting prey = 0.5). Probit regression is often used to estimate the median lethal dose (the dose which has been found to be lethal to 50 % of test subjects) of a compound (Williams 1986; Takeuchi and Endo 2012; Sáez et al. 2013). Similarly to lethal dose, prey detectability has been found to be exponential in numerous studies and probit regression is considered to be the appropriate analysis for this type of data (Chen et al. 2000; Payton et al. 2003; Greenstone et al. 2007; Greenstone et al. 2010). It is important to note that this analysis measured the detectability of frogs as prey over time (i.e. present/absent for each sample) and was not necessarily a measure of prey identifiability (e.g. the degradation of prey items over time).

Paired-sample t-tests were used to compare pre-ingestion maximum bone length (tibiofibula) with post-ingestion maximum bone length (longest bone in stomach sample). Independent t-tests were used to determine whether there was a significant difference in this reduction of prey bone length (tibiofibula – longest bone in stomach sample) between species. Reduction in prey bone length from all euthanasia intervals were grouped for predator species as otherwise there would have been too few data for the analysis. This was considered appropriate as maximum bone length did not differ significantly between intervals (see Section 3.3.4). Where variances were unequal, an unequal variance t-test was used (Ruxton 2006).

Analysis of variance (ANOVA) was used to compare the mean ingestion rate and maximum prey bone length between species. The Levene’s test for homoscedasticity (Levene 1960) revealed that some groups had significantly differing variances. Therefore the Games-Howell
post hoc test for unequal variances was applied (Games and Howell 1976). To investigate the relationship between body mass and ingestion rate, data was log (ln) transformed and linear regression used (Shipley et al. 1994). Linear regression was also used to investigate the relationships between ingestion rate (g/min), reduction in prey bone length (mm), and the frog mass (g):predator mass (g) ratio.

To investigate the effects of digestion on prey bone length a linear model approach was used. All models were run in R (R Core Team 2013), using the packages nlme (Pinheiro et al. 2013), MASS (Venables and Ripley 2002), nnet (Venables and Ripley 2002) and car (Fox and Weisberg 2011). The Shapiro-Wilk normality test was used to ensure prey bone lengths were from a normal distribution (Shapiro and Wilk 1965). Where data were not normal a Box-Cox transformation was applied (Box and Cox 1964). A p-value of < 0.05 was considered significant for all tests. For the stomach content data set a general linear model was used with species, time since prey ingestion, and ingestion rate as fixed factors. Species was always a categorical factor and ingestion rate always a continuous factor. Two different sets of models were run using time since prey ingestion as 1) a continuous factor and 2) as a categorical factor. A very similar model was used for the faecal content data set, but this included animal ID as a random factor, to account for the multiple observations from one subject, and as such this was a linear mixed effects model. Faecal content data were fitted using the maximum likelihood method, as this is a more suitable method for comparing different mixed effects models with varying fixed effects. Prior to model running all continuous variables were z-transformed. For each of the two data sets, the most complex model possible (including all factors and interactions) was run. A stepwise model selection by Akaike Information Criterion (AIC) was carried out using the stepAIC function and the model with the lowest AIC was selected for final interpretation of results.
A generalised estimating equation (GEE) was used to investigate whether any of the following variables had an effect on the successful identification of frogs as prey (to order level): ingestion rate, maximum prey bone length, predator mass, frog mass:predator mass ratio. The model used a logit link with species as a factor, collection time (h) as a repeated measure within individual ID, prey detection (yes/no) as a binomial dependent variable and maximum prey bone length, predator mass and frog mass:predator mass ratio as independent predictors (Liang and Zeger 1986; Ballinger 2004). Model selection was based on the extension of the Akaike Information Criterion for generalised estimating equations developed by Pan (2001): the quasi-likelihood under independence model criterion (QIC).

Ship rats and mice were excluded from analyses involving prey bone length reduction, as they consumed only partial frogs during feeding trials (and mice did not consume any bones prior to euthanasia or faecal collection trials; see Chapter 2 for details on frog body parts chosen by respective small mammal species).

3.3 Results

3.3.1 Effect of washing on hedgehog GIT transit

As would be expected, time had a significant effect on hedgehog GIT transit (Greenhouse-Geisser correction applied; $F_{1, 1.043} = 16.06, p < 0.005$). However, the interaction washing*time did not (Greenhouse-Geisser correction applied; $F_{1, 1.043} = 0.198, p = 0.68$). The large difference in $F$–values for these factors strongly indicates that the washing procedure did not significantly affect the faecal collection during these experiments. As a result this procedure was used for all subsequent faecal collection for hedgehogs. Of note is the relatively fast total GIT transit observed, with the majority of the plastic pellets expelled within 6 to 9 h post-ingestion and the maximum time taken to expel plastic pellets being 12 h (Figure 3.2).
Figure 3.2 The percentage of plastic pellets observed in hedgehog faeces during 4 collections spaced 3 h apart following plastic pellet ingestion. By 12 h, 100% of plastic pellets had been recovered. There was no significant difference between hedgehogs subject to washing (n = 6, ■) and control group (n = 6, □). Error bars indicate standard error.

3.3.2 Identification of frog prey remains

3.3.2.1 Stomach contents

The proportion of stomach samples that contained identifiable prey remains was very low (Table 3.4). It was not possible to identify any frog remains in mouse stomach contents because they consumed no skeletal components in the trials preceding euthanasia. In only three cases was it possible to identify frog remains to genus or species level (all in hedgehog stomach contents).
Table 3.4 Number and proportion (%) of stomach samples observed to have identifiable frog remains to order level or below (obs<sub>order</sub>), genus level or below (obs<sub>genus</sub>), or species level (obs<sub>species</sub>). Also shown are the number of subjects within each euthanasia time interval (n).

<table>
<thead>
<tr>
<th>Species</th>
<th>n, obs, %</th>
<th>1</th>
<th>3</th>
<th>6</th>
<th>9</th>
<th>12</th>
<th>Overall</th>
</tr>
</thead>
<tbody>
<tr>
<td>laboratory Norway rat</td>
<td>n</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>3</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>obs&lt;sub&gt;order&lt;/sub&gt;</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>-</td>
<td>2.0</td>
</tr>
<tr>
<td></td>
<td>%</td>
<td>2.0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>-</td>
<td>5.56</td>
</tr>
<tr>
<td>ship rat</td>
<td>n</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>obs&lt;sub&gt;order&lt;/sub&gt;</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>6.67</td>
</tr>
<tr>
<td></td>
<td>%</td>
<td>0</td>
<td>0</td>
<td>2.0</td>
<td>-</td>
<td>-</td>
<td>6.67</td>
</tr>
<tr>
<td>mouse</td>
<td>n</td>
<td>5</td>
<td>6</td>
<td>4</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>obs&lt;sub&gt;order&lt;/sub&gt;</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>-</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>%</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>-</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td>hedgehog</td>
<td>n</td>
<td>4</td>
<td>6</td>
<td>7</td>
<td>4</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>obs&lt;sub&gt;order&lt;/sub&gt;</td>
<td>3</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>%</td>
<td>75.0</td>
<td>16.67</td>
<td>14.29</td>
<td>0</td>
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<td>%</td>
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<td></td>
<td>%</td>
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</tbody>
</table>

Due to the high number of empty cells for rodent species (i.e. groups that contained zero positive responses), a meaningful probit regression was only possible for hedgehogs. The probit model fitted the hedgehog data well ($\chi^2_{3,n=24}=1.58, p=0.66$) and the slope of the prey detection rate was significantly different from zero ($Z=-2.05, p=0.04$; Figure 3.3), indicating prey detectability declined over time. The half-life of prey detection was 1.77 h (95 % fiducial limits: 0 h, 3.85 h). A complete list of all prey items observed in stomach contents is provided in Appendix D.
Figure 3.3  Probit regression showing the probability of detecting frog remains to order level in the stomach contents of hedgehogs using morphological identification techniques. Actual data is plotted (■), solid line shows fitted probit regression. Dashed lines indicate upper and lower 95% fiducial limits from 0 – 12 h.

Muscle and cartilaginous tissue was present in many of the stomach samples, but did not aid taxonomic identification in any way. Although skeletal components were observed (see Figure 3.4 and Appendices D and F), and sometimes included bones that are used in distinguishing between anuran genera (such as the ilium; Bever 2005), they were often highly degraded, making it impossible to discern traits diagnostic of genus or species. It should also be noted that a number of the samples contained intact invertebrate material, originating from insects fed to southern bell frog individuals prior to euthanasia. This is testament to the durability of chitin, evidence for the detection of secondary predation and demonstrates the relative perishability of frog prey components.
3.3.2.2 Faecal contents

The proportion of faecal samples that contained identifiable prey remains was even lower than it was for stomach samples (Table 3.5). It was not possible to identify any frog remains in mouse stomach contents because they consumed no skeletal components in the trials preceding faecal collection. However, it was also not possible to identify any frog remains in either rat species, both of which had consumed frog bones. This is in contrast to hedgehog faeces which often contained identifiable prey remains, sometimes to genus level (Table 3.5; see Figure 3.5 and Appendices E and F). It was not possible to identify any frog remains to species level.
Table 3.5 Number and proportion (%) of faecal samples observed to have identifiable frog remains to order level or lower (obs\textsuperscript{order}), or to genus level (obs\textsuperscript{genus}). Also shown are the number of subjects that defecated within each faecal collection time interval (n).

<table>
<thead>
<tr>
<th>Species</th>
<th>n, obs, %</th>
<th>Hours since frog ingestion</th>
<th>% Overall</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>6</td>
<td>12</td>
</tr>
<tr>
<td>laboratory Norway rat</td>
<td>n</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>obs\textsuperscript{order}</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>%</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>ship rat</td>
<td>n</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>obs\textsuperscript{order}</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>%</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>mouse</td>
<td>n</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>obs\textsuperscript{order}</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>%</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>hedgehog</td>
<td>n</td>
<td>13</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>obs\textsuperscript{order}</td>
<td>10</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>%</td>
<td>76.92</td>
<td>50.0</td>
</tr>
<tr>
<td></td>
<td>obs\textsuperscript{genus}</td>
<td>6</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>%</td>
<td>46.15</td>
<td>33.33</td>
</tr>
</tbody>
</table>

As for stomach contents, the high number of empty cells for rodent species (i.e. groups that contained zero positive responses), meant that a meaningful probit regression was only possible for hedgehogs. The probit model fitted the data well (Pearson $\chi^2$\textsubscript{3, n = 49} = 0.53, $p = 0.91$), and the prey detection rate differed significantly from zero ($Z = -3.59$, $p < 0.001$; Figure 3.6). The half-life for detecting frog prey in hedgehog faecal contents was 10.73 h (95 % fiducial limits: 6.61 h, 13.8 h).

Muscle and cartilaginous tissues were present in relatively few faecal samples compared to stomach samples. Skeletal components were present to a similar degree, usually highly degraded, as for stomach contents.
Figure 3.5 Examples of prey components found in small mammal faecal contents. A – Radioulna, identifiable to genus level (in hedgehog faeces, 12 h post-ingestion); B – Femurs, identifiable to genus level (in hedgehog faeces, 6 h post-ingestion). A more comprehensive photographic index is provided in the Appendix E.

Figure 3.6 Probit regression showing the probability of detecting frog remains to order level in the faecal contents of hedgehogs using morphological identification techniques. Observed data is plotted (■), solid line shows fitted probit regression, dashed lines indicate upper and lower 95 % fiducial limits.
3.3.3 Effect of ingestion on prey bone length

The ingestion of frogs significantly reduced frog maximum bone length by 75 % and 64 % in laboratory Norway rats and hedgehogs respectively (Table 3.6). This reduction was not significantly different between the two species ($t_{11.75, n = 28} = 0.83$, $p > 0.4$; unequal variance $t$-test). It was not possible to investigate bone reduction for mice or ship rats as they consumed only partial frogs during feeding trials.

Table 3.6 Maximum prey bone length (mm) pre-ingestion (tibiofibula) and post-ingestion (longest bone in predator stomach sample). Also shown are mean differences (mm), associated standard errors, $t$-values, degrees of freedom (df) and $p$-values, based on paired sample $t$-tests.

<table>
<thead>
<tr>
<th>Species</th>
<th>n</th>
<th>Pre-ingestion</th>
<th>Post-ingestion</th>
<th>Mean difference</th>
<th>SE</th>
<th>$t$</th>
<th>df</th>
<th>$p$</th>
</tr>
</thead>
<tbody>
<tr>
<td>laboratory Norway Rat</td>
<td>17</td>
<td>12.85</td>
<td>3.27</td>
<td>9.58</td>
<td>0.42</td>
<td>-22.76</td>
<td>16</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>hedgehog</td>
<td>11</td>
<td>13.05</td>
<td>4.71</td>
<td>8.34</td>
<td>1.43</td>
<td>-5.817</td>
<td>10</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>

The ingestion rate of mammal groups differed significantly ($F_{3, n = 62} = 41.13$, $p < 0.001$; Figure 3.7). Only ship rats and laboratory Norway rats exhibited similar ingestion rates (Games-Howell post hoc comparison, $p > 0.9$). Although the reduction in prey bone length was substantial, the difference in resultant post-ingestion maximum prey bone lengths between species was not significant ($F_{2, n = 84} = 1.7$, $p > 0.15$).
Ingestion rate was found to scale with body mass with the relationship: Ingestion rate = (0.007)Body mass$^{0.69}$ (Figure 3.8). However, ingestion rate did not correlate with reduction in bone length ($R^2 = 0.07, F_{1,n=24} = 1.61, p > 0.2$), and neither did body mass ($R^2 = 0.13, F_{1,n=28} = 4.0, p > 0.05$). This indicates that while the ingestion of frogs as prey had a very strong effect on the reduction of prey bone length, the rate at which prey were ingested did not. It should be noted that sample sizes were low, due to the low number of samples in which bones were found.

In laboratory Norway rats, the ratio of frog mass:predator mass had a significant (although weak) effect on the reduction of bone length ($R^2 = 0.6, F_{1,n=17} = 22.18, p < 0.001$; reduction in prey bone length = 11.51 - 0.008[frog mass:predator mass]) and ingestion rate ($R^2 = 0.65, F_{1,n=14} = 22.62, p < 0.001$; ingestion rate = 0.19 - 0.0002[frog mass:predator mass]).
hedgehogs, neither variable was correlated with the ratio of frog mass:predator mass (reduction in prey bone length: \( R^2 = 0.006, F_{1, n=11} = 0.05, p > 0.8 \); ingestion rate: \( R^2 = 0.03, F_{1, n=10} = 0.22, p > 0.6 \)).

![Figure 3.8 Body mass versus ingestion rate for laboratory Norway rats (■), ship rats (○), mice (●) and hedgehogs (♦). Linear regression line is indicated, \( R^2 = 0.47 \).](image)

3.3.4 Effect of digestion on prey bone length

Treating time as either a continuous or a categorical factor did not change which variables had a significant effect on maximum prey bone length. The general results are presented here, but see Appendix G for more details of model selections and results obtained. Maximum prey bone length did not change over time (since frog ingestion) in stomach contents for any of the species \((p > 0.1)\), while in faecal contents it came close to being significantly affected \((t_8 = -2.22, p = 0.057)\), suggesting that longer duration in the gut of the predator may cause a minor
decrease in prey bone length, but higher numbers of samples containing bones would be required to confirm this.

3.3.5 Influence of bone length on identification of frogs as prey

The bones used to identify frogs as prey ranged in length from 1.6-12 mm in stomach contents and 1.05 to 14.67 mm in faecal contents. The generalised estimating equation model that best explained the variation of the data included all variables as predictors and resulted in maximum prey bone length being a significant factor affecting successful prey identification. Neither predator mass (Wald $\chi^2_{1, n=65} = 2.14, p > 0.1$), frog mass:predator mass ratio (Wald $\chi^2_{1, n=65} = 1.62, p > 0.2$), nor ingestion rate (Wald $\chi^2_{1, n=65} = 3.5, p = 0.06$) were significant factors. The model predicts that for every increase of 1 mm prey bone length, the chance of identifying frogs as prey (to order level) will be 4.69 times as likely (Wald $\chi^2_{1, n=65} = 4.65, p = 0.03$; lower 95 % CI 1.15, higher 95 % CI 19.09).

All the variables and inter-relationships investigated in the present study, and their association with the successful identification of frogs as prey (to order level) are represented in Figure 3.9.
3.4 Discussion

Overall this chapter has demonstrated the difficulties involved with identifying frogs as prey in small mammal diet samples. The primary finding of this study is that, in general, the success rate of identifying frogs as prey is low, particularly to species level, even relatively soon after rodents or hedgehogs have consumed an entire frog. Evidence from the literature suggests that identifying amphibian remains often poses difficulties (e.g. Coman 1973; Roser and Lavers 1976; Chanin and Linn 1980; Webb et al. 1982; Nilsson 1984; Weber 1990; Harna 1993; King 1993; Martin et al. 1996; Barratt 1997; Ruiz-Olmo and Palazón 1997; de Queiroz et al. 2001; Reif et al. 2001; de Aguiar and Di-Bernardo 2004; Hammershøj et al. 2004; Clavero et al. 2005; Britton et al. 2006; Żmihorsk and Osojca 2006; Kutt 2011).
Britton et al. (2006) were, for the vast majority, unable to identify beyond class level of amphibian prey in otter stomach contents, even though amphibians were present in 25% of the samples. Coman (1973) could not identify frogs beyond order level in red fox (Vulpes vulpes) stomach contents in Australia. Roser and Lavers (1976) were unable to distinguish between brown tree frogs and southern bell frogs (species notably different in size) in ferret (Mustela putorius furo) scats where anuran remains were noted in 17% of samples. Beard and Pitt (2006), following a diet study of ship rats in Hawaii, concluded that no rats had consumed Coquí frogs (Eleutherodactylus coqui). However, in one instance during fieldwork in the same study area, a ship rat was observed in possession of a Coquí frog (Woolbright et al. 2006). While it may have been the case that none of the rats studied had consumed frogs during the study period, it is also a possibility that rats were consuming viscera and muscle tissue and very little or no skeletal components, which would have resulted in false negatives. This poses a major problem to investigators interested in quantifying small mammal predation on frogs.

### 3.4.1 Effects of ingestion and digestion on maximum prey bone length

Overall the results of this study show that the breakage effect produced by mastication of frog prey is much larger than the effects of digestion in small mammals. When analysing field-collected scat samples of various mammals, Pinto Llona and Andrews (1999) observed that larger frogs underwent mastication whereas smaller individuals were swallowed whole, preserving the bone structures more completely. In the present study, the ratio of frog mass:predator mass did not affect the reduction in prey bone size as might have been predicted, based on Pinto Llona and Andrews (1999). This is likely because only laboratory Norway rats and hedgehogs were of similar body mass and ship rats and mice could not be included in the analyses. Pinto Llona and Andrews (1999) concluded that mammalian predators produce strong bone breakage effects and relatively weaker digestive effects on amphibian bones (Pinto Llona and Andrews 1999) and this is supported by the present study.
The relationship between mammalian ingestion rate and body mass with a scaling power of 0.69, is similar to that found in other studies (0.69 in Gould 1975; 0.7 in Shipley et al. 1994). Ingestion rate is directly proportional to chewing frequency (chews/min) and chewing intensity (chews/g; Shipley et al. 1994), but in the present study, maximum prey bone length did not differ between predator species, and was not significantly affected by ingestion rate. Prey bone length directly affected the successful identification of frogs as prey. However, because very small bones were often diagnostic of frogs as prey (e.g. vertebrae), this does not appear to always be a defining factor.

Gastric emptying half-lives have been reported for laboratory Norway rats as c. 1.6 h (Droppleman et al. 1980; Enck et al. 1989), although complete stomach emptying in ship rats was observed to take over 6 h (Weld et al. 2004), while total GIT transit (ingestion to defecation) is estimated at 15 – 22 h (Sigleo et al. 1984; Enck et al. 1989). The extremely low proportions of samples in which frogs could be detected as prey (even to order level) in rodent stomachs (0 – 7 %) or faeces (0 %) across all time intervals, suggests that it is not movement of prey through the GIT that affects the identification of prey, but the initial effects of ingestion and bone breakage. In hedgehogs, GIT transit has been previously been estimated as 12 – 16 h (Reeve 1994), although in the present study was observed to be 6 – 12 h. Hedgehog stomach emptying times have not been reported, but given the relatively short GIT, they are likely to be quite short too. Frogs could be identified as prey (with a probability of 0.5) at 1.77 h and 10.73 h in hedgehog stomach and faecal contents respectively, suggesting that in this case, movement of prey through the GIT, as well as initial bone breakage, affected the prey detection rate.

3.4.2 Techniques used

It may have been possible, using different morphological based techniques, such as scanning electron microscopy, which has been used to inspect amphibian remains from predator
stomach and faecal samples (Worthy and Holdaway 1994; Pinto Llona and Andrews 1999), or microtomography, which has been used to obtain detailed images of amphibian bones (Biton et al. 2013; Sanchez et al. 2013), to attain a higher degree of success in taxonomic identification. This would necessarily involve a higher degree of expertise, resources and time on the investigator’s part.

Measuring average bone size in each diet sample may have clarified the relationship between ingestion rate and prey bone size, but this was not possible because many of the (very numerous) smaller shards and fragments could not be unambiguously identified as bone. It may have been possible to do this using various staining techniques, such as those used on frog skeletons (Green 1952) and for distinguishing rodent diet sample contents (Harris 1986). However, staining procedures would have required a number of chemicals to be added to the diet samples, such as hydrogen peroxide, formaldehyde, alizarin red, alcian blue and/or sulphuric acid (reviewed in Clark 1973). Such additions are likely to have affected the downstream molecular analyses for these samples as they may damage DNA (as hydrogen peroxide is known to do; Kahnert et al. 2005) or inhibit the PCR reaction (as formaldehyde is known to do; reviewed in Schander and Kenneth 2003). Because the samples were already likely to contain heavily degraded DNA from the digestion process (reviewed in Symondson 2002 and; King et al. 2008) and contain known PCR inhibitors such as calcium (Opel et al. 2010) and bile salts (Lantz et al. 1997), the addition of further materials that may have interfered with downstream processes was not viable in the present study.

3.4.3 Observer experience

This study was approached as an investigation into techniques that may be applied by a range of researchers. Although I had previous experience with diet analysis and frog morphology, I was not highly specialised in the identification of anuran bones. It is possible that an investigator with extensive experience in frog bone identification would have had a higher
success rate, and visual observation always depends on the skills of the observer (Symondson 2002; Soininen et al. 2009; Pompanon et al. 2012). However, any success rate derived from such data could not be applied to the more general amphibian researcher. Even so, investigators that are experienced in diet analysis have reported difficulties in identifying prey components in small mammal stomach samples (Hansson 1970; Miller and Miller 1995). Where it is possible, a considerable amount of time is required to identify all food items in a rat stomach; Clark (1982) reported times of 3 – 4 h per stomach sample to identify all material, and in the present study, focusing only on frogs as prey, each sample took c. 0.5 h to process. Rodent stomach contents are notoriously difficult to identify, due to the level of mastication effected by this group (Hansson 1970). While this may have affected overall success, the fact that the same observer inspected all samples means that results among samples within this study are still directly comparable.

3.4.4 Prey items investigated

The prey bones (and in rare cases prey external features) used in this study for identification to various taxonomic levels were chosen based on previous papers that utilised such bones for species and/or genera differentiation. As samples were treated as if they had been collected from unknown locations in mainland New Zealand, there were five possible frog species to distinguish between. In the genus *Leiopelma* are Archey’s frog and Hochstetter’s frog, both of which are relatively small amphibians and possess many skeletal components that may remain cartilaginous throughout adulthood (Stephenson 1960). A sexually mature specimen of Archey’s frog is typically comparable with an immature form of Hochstetter’s frog as far as size and skeletal characters are concerned (Stephenson 1960). This immediately negates the ability of researchers to identify frogs to species level. Hochstetter’s frog occurs sympatrically with all known Archey’s frog populations (Thurley and Bell 1994a). Similarly, southern bell frog and green and golden bell frog skeletons are not likely to be readily distinguishable in predator stomach contents where these species co-exist. Skull bones can be
useful in discerning frogs to species level, but very few of these were observed in this study and a high level of experience would be required to make judgements on these bones.

Looking at fossil frogs, Trueb (1973) noted that in many cases identifying anura to taxonomic levels within the order is often difficult due to the osteological plasticity within many groups.

3.4.5 Conclusions

This is the first study to investigate the morphological detection and identification of frogs as prey in small mammal stomach and faecal contents under laboratory conditions. It concludes that these traditional techniques are of limited use, considering the difficulties in identifying frog bones, the effects of ingestion and the fact that small mammals often do not ingest the bones of frogs. Initial breakage effects during ingestion of frogs appear to be a major limiting factor, while the effects of digestion are relatively weaker. Although very small bones can be used to identify frogs as prey, the odds of successful identification dramatically increase as maximum prey bone length increase.
Chapter 4  DNA-based diet analysis to detect frogs as prey in small mammal stomach and faecal samples

Abstract

Wildlife management decisions regarding the control of introduced predators to protect endangered species are often based on predation rates derived from diet studies of the predators concerned. To gather this important information from diet studies, it is essential that techniques exist to reliably detect prey remains. Morphological techniques to identify frogs as prey in small mammal stomach and faecal samples are unreliable because small mammals often do not consume skeletal material when ingesting frogs, or in cases where they do ingest bones, these are often too heavily masticated to allow identification of the prey species. The aim of this study was to develop and assess the use of novel DNA-based methods to detect frogs as prey in introduced small mammal diets. Species-specific primers were developed to detect the presence of DNA from three frog species in New Zealand (Leiopelma archeyi, Leiopelma hochstetteri and Litoria raniformis). Stomach and faecal samples of four small mammal species were collected following the ingestion of frogs under laboratory conditions. DNA-based methods far outperformed traditional morphological diet analysis and prey detection periods exceeded the average small mammal gastrointestinal transit times. This is the first time that prey DNA detectability over time has been measured for small mammal stomach contents and the first time that prey DNA detectability has been compared over time, between stomach and faecal contents, in any vertebrate. The result is a valid ecological tool that can be easily adapted to other diet analysis studies. This has major implications for both global amphibian conservation and small mammal diet analysis in general.
4.1 Introduction

Morphological techniques to identify frogs as prey in small mammal stomach and faecal samples are unreliable because small mammals often do not consume skeletal material when ingesting frogs (Chapter 2), or in cases where they do ingest bones, these are often too heavily masticated to allow identification of the prey species (Chapter 3). Furthermore, even when complete frog bones are found in predator diets, they are not always diagnostic of the frog species. To use a New Zealand example, Archey’s frog (*Leiopelma archeyi*) and Hochstetter’s frog (*Leiopelma hochstetteri*) are both relatively small amphibians that occur sympatrically and possess many skeletal components that may remain cartilaginous throughout adulthood (Stephenson 1960). Stephenson (1960) made particular note that a sexually mature specimen of Archey’s frog is typically comparable with an immature form of Hochstetter’s frog as far as size and skeletal characters are concerned, which would make it prohibitively difficult to distinguish between these species as prey in predator stomach or faecal samples.

Molecular analysis of predation, i.e. polymerase chain reaction (PCR) amplification of prey DNA within the faeces or digestive systems of predators, is a rapidly growing field (King et al. 2008), which is used to study complex trophic interactions. DNA identification allows wildlife managers to detect the presence of endangered taxa and manage their conservation (Farrell et al. 2000). In many cases, morphological analyses are simply not possible, for example, most invertebrates as predators are fluid feeders (Sunderland 1988; Symondson 2002; Admassu et al. 2006; Greenstone et al. 2007; Pompanon et al. 2012) that consume no identifiable prey components, precluding morphological analysis. Even when morphologically identifiable remains of prey are present in predator faeces or stomachs, DNA analyses have been shown to detect prey more frequently and accurately, in some cases increasing prey detection by 20 – 37 % (Casper et al. 2007b).
Chapter 4 – Detecting frogs as prey using DNA-based analysis

Research to date in this field, particularly into the reliability and sensitivity of PCR to detect prey, has largely been carried out in invertebrates, as both predators and prey (reviewed in Symondson 2002; Sheppard and Harwood 2005; and King et al. 2008). While DNA-based prey identification techniques have often been applied to stomach or faecal samples collected in vertebrate field studies (Höss et al. 1992; Taberlet and Fumagalli 1996; Reed et al. 1997; Scribner and Bowman 1998; Farrell et al. 2000; Nelson et al. 2000; Jarman et al. 2002; Symondson 2002 [review]; Jarman et al. 2004; Jarman and Wilson 2004; Lee et al. 2006; Bradley et al. 2007; Deagle et al. 2007; Clare et al. 2009; Meekan et al. 2009; Soininen et al. 2009; Tollit et al. 2009; Corse et al. 2010; Dunn et al. 2010; Riemann et al. 2010; Zeale et al. 2010; Rayé et al. 2011), there have been relatively few studies on the decay rate of prey detectability over time. For invertebrate studies, a common method to assess the efficacy of DNA-based diet techniques is to feed invertebrate prey to invertebrate predators, to subsequently euthanize predators at suitable time intervals, and then carry out PCRs targeting prey DNA in predator gut, faecal or whole body samples to determine the decay rate of detection probability (e.g. Asahida et al. 1997; Agustí et al. 1999; Agustí et al. 2000; Chen et al. 2000; Symondson 2002 [review]; Greenstone et al. 2007; Weber and Lundgren 2009; Greenstone et al. 2010). For vertebrates as predators, this type of prey DNA detection over time since prey ingestion has rarely been carried out (Rosel and Kocher 2002; Deagle et al. 2005b; Casper et al. 2007b; Carreon-Martinez et al. 2011; Oehm et al. 2011; Hunter et al. 2012) and has never been reported for mammal stomach contents. Furthermore, no study has compared stomach versus faecal prey DNA detectability over time since prey ingestion in any vertebrate predator.

Within the discipline of molecular diet analysis, a critical step is the selection of suitable primer pairs for targeting specific prey or prey groups (Zarzoso-Lacoste et al. 2013), and the primers used will depend on the information being sought. There are two main approaches that can be used here: 1) a group-specific approach and; 2) a species-specific approach.
Group-specific primers are designed to identify sites that are conserved within a group (e.g. class, order or genus), but unique between groups (King et al. 2008). Species-specific primers are designed to amplify the target DNA from only one prey species. Both approaches have been used successfully in many studies (reviewed in Symondson 2002; Sheppard and Harwood 2005; and King et al. 2008). The group-specific approach may involve more resource-consuming analyses as amplified products from multiple prey species may require separation by cloning prior to sequencing (e.g. Agustí et al. 1999; Agustí et al. 2000; Deagle et al. 2005b; Zeale et al. 2010; Zarzoso-Lacoste et al. 2013) or the application of relatively expensive next-generation sequencing (Clare et al. 2009; Bohmann et al. 2011; Murray et al. 2011; Brown et al. 2012; Pompanon et al. 2012 [review]; Boyer et al. 2013). One primary objective of the research in this thesis is the assessment of the impact of generalist predatory small mammals on three frog species (southern bell frog *Litoria raniformis*, Archey’s frog and Hochstetter’s frog; see Chapter 5). A species-specific primer approach is suitable for this objective as there are few prey species being targeted.

Hence the aims of the research presented in this chapter were:

1. To develop species-specific primers, and associated PCR protocols, for Archey’s frogs, Hochstetter’s frogs and southern bell frogs.

2. To compare the efficacy of two DNA extraction techniques when extracting prey remains from small mammal stomach contents: Chelex extraction and the Qiagen ‘DNAeasy blood and tissue’ kit.

3. To quantify the decay rate of the probability of detecting frogs as prey using molecular methods in small mammal stomach and faecal contents.
4.2 Methods

4.2.1 Stomach and faecal sample collection

Animal sourcing, animal husbandry and feeding trial procedures are detailed in Chapters 2 and 3 of this thesis. A summary of the groups and the prey consumed prior to either faecal collection or euthanasia is provided in Table 4.1. Greenstone et al. (2013) expounded the importance of feeding the same quantity of prey to each predator when estimating half-life detectability and indicate the assumption is met if the prey item is small enough that a predator can consume all of it in one sitting. This was true for hedgehogs (Erinaceus europaeus) and laboratory Norway rats (Rattus norvegicus), so samples obtained from feeding trials detailed in Chapter 2 were used for downstream analysis here. Mice (Mus musculus) consume skeletal elements in only 38% of cases when presented entire frogs (Chapter 2), which may influence DNA detectability, so to standardise their ingestion of frog material they were presented 0.1 g of frog muscle.

Wild ship rats (Rattus rattus) were presented entire frogs but ingested different body parts in varying proportions. The fact that wild ship rats often consume only soft tissue when presented entire frogs (Chapter 2) may affect the detection rate of prey DNA in stomach or faecal samples. The number of wild ship rats included in this study did not allow for an investigation of this directly, so laboratory Norway rats were used as a model species. Subjects were either presented entire frogs (see above) or a 2 g mix of frog soft tissue in the following proportions: leg muscle (51%), liver (10%) and gastrointestinal tract (GIT; 39%). A combination of different tissues was used as the number of mitochondria (containing the target gene used in this study, see Section 4.2.3) per cell in each tissue type varies widely (Alberts et al. 1994). The proportions of each tissue type used reflect those present in southern bell frogs. This was calculated by removing the muscle, liver and GIT of 10 southern bell frogs and taking the mean weight of each tissue type (data not shown).
Table 4.1 Summary of the number of animal subjects within each group, prey material ingested, and type of sample collected following prey ingestion.

<table>
<thead>
<tr>
<th>Species</th>
<th>Prey parts ingested</th>
<th>Sample type</th>
<th>n subjects (n faecal samples)</th>
</tr>
</thead>
<tbody>
<tr>
<td>laboratory Norway rat</td>
<td>entire frog</td>
<td>stomach</td>
<td>18</td>
</tr>
<tr>
<td>laboratory Norway rat</td>
<td>frog soft tissue – 2 g</td>
<td>stomach</td>
<td>23</td>
</tr>
<tr>
<td>hedgehog</td>
<td>entire frog</td>
<td>stomach</td>
<td>24</td>
</tr>
<tr>
<td>ship rat</td>
<td>frog parts as chosen by subject</td>
<td>stomach</td>
<td>15</td>
</tr>
<tr>
<td>mouse</td>
<td>frog muscle – 0.1 g</td>
<td>stomach</td>
<td>15</td>
</tr>
<tr>
<td>laboratory Norway rat</td>
<td>entire frog</td>
<td>faeces</td>
<td>6 (24)</td>
</tr>
<tr>
<td>laboratory Norway rat</td>
<td>frog soft tissue – 2 g</td>
<td>faeces</td>
<td>9 (36)</td>
</tr>
<tr>
<td>hedgehog</td>
<td>entire frog</td>
<td>faeces</td>
<td>16 (49)</td>
</tr>
<tr>
<td>ship rat</td>
<td>frog parts as chosen by subject</td>
<td>faeces</td>
<td>10 (41)</td>
</tr>
<tr>
<td>mouse</td>
<td>frog muscle – 0.1 g</td>
<td>faeces</td>
<td>10 (49)</td>
</tr>
</tbody>
</table>

In order to detect whether any systematic flaws existed that may have led to contamination during any stage of the procedures, the faecal and stomach samples from one subject of each species were collected after they had consumed no frog prey whatsoever. These samples were treated in an identical manner through all protocols as those from subjects that had consumed frog material. All stomach and faecal samples were stored at room temperature in 95 % ethanol for 5 – 28 months prior to DNA extraction.

4.2.1.1 Hedgehog faecal collection

As noted in Chapter 3 of this thesis, it was not effective to use a raised wire mesh floor for hedgehog cages, due to the viscous consistency of the hedgehog faeces, which caused it to stick to the wire mesh and the underside of the animal. In order to combat this, hedgehogs were briefly immersed in a series of 4 warm water baths between trials to remove any residual faeces before being transferred into a new cage. This did not have a significant effect on GIT transit time (Chapter 3), but to assess whether there was any carryover of material from one cage to the next, between faecal collection intervals, a worst-case scenario washing experiment was undertaken. Each hedgehog (n = 4) was smeared on its underside with 2.5 g of homogenised frog viscera and muscle (the mean weight of the frogs being fed to
hedgehogs). Washing was then applied in the 4 successive warm water baths, before placing the hedgehog in a new cage. In order to collect all solid material, the water from each final bath was collected and centrifuged at 13,000 x G for 20 minutes in six 250 ml centrifuge containers. The majority of the supernatant was decanted and the remaining water (c. 8 ml) from each container was combined into a new 50 ml centrifuge tube. This was again centrifuged at 5,000 x G for 10 minutes, in order to form a pellet of solid material. The supernatant was decanted and the pellet was resuspended in 1 ml DNAase-free filtered water (Sigma-Aldrich) in a 1.5 ml centrifuge tube and stored at -20 °C until ready for DNA extraction and subsequent PCR to assay for the presence of frog DNA. The samples were subjected to the same extraction and PCR protocol as for all other faecal samples collected (described in Sections 4.2.2 and 4.2.5).

4.2.1.2 Homogenising

Stomach and faecal contents were homogenised using a rotor-stator homogeniser (IKA Ultra-Turrax T25; IKA-Labortechnik) with a 10 mm diameter plastic homogenising probe (S 25 D 10 G – KS; IKA-Labortechnik) at 8,000 RPM. This was carried out for two reasons: 1) it removed any potential subsample selection bias during the downstream DNA extraction stage (Rosel and Kocher, 2002 and Deagle et al., 2005b found that blended samples yielded a higher prey detection frequency than subsamples) and; 2) it provided a mechanical breakdown of hard and soft tissue in the samples, potentially aiding the downstream DNA extraction process. To minimise the carryover of material between samples the following protocol was followed for each sample: 1) probe soaked in 20 % commercial bleach (sodium hypochlorite) for 30 minutes; 2) probe rinsed by hand in two successive tap water baths; 3) probe attached to homogeniser and run in two successive falcon tubes of 100 % ethanol for 15 s to remove residual bleach (immersed to the maximum limit; Aslanzadeh 2004); 4) probe run in falcon tube of tap water for 15 s; 5) probe run in sample for up to 30 s; 6) probe
removed from homogeniser and dismantled into component parts; 7) component parts rinsed in tap water bath; 8) probe reassembled and placed in 20 % bleach. This method was based on results from Bonne et al. (2008) who found that agitating contaminated equipment in 100 % bleach (for 5 s), then in tap water, and finally in 100 % ethanol, produced no detectable carryover in subsequent samples. Only 20 % bleach was used in this protocol, as Prince and Andrus (1992) demonstrated that a 10 % bleach treatment prevented amplification of a 600 bp DNA fragment within one minute of exposure. Plastic probes were found to be advantageous because, unlike stainless steel probes, these can be immersed in bleach without causing them damage. In addition, the stator shafts are transparent, allowing the observer to easily inspect for any debris or material that may be stuck in the shaft. With multiple probes it was possible to immerse each one in bleach for 30 minutes, while simultaneously continuously processing samples. However, they did tend to break on very tough samples that contained thick bones, so may not be suitable for all sample types.

To ensure that this protocol did indeed remove carryover contamination, and did not negatively affect DNA amplification, a worst-case scenario experiment was carried out.

1. The homogenising probe was immersed and run in a sample containing 2 g of fresh frog tissue (including skin, muscle, viscera and bone) in 30 ml 95 % ethanol (a similar volume to samples collected) until the sample was fully homogenised (c. 25 s). The probe was then run for 25 s in 3 ml DNase-free water (Sigma-Aldrich). This was repeated for 5 separate samples. Each 3 ml water sample (n = 5) was stored at -20 °C until it could be subjected to DNA extraction (using the ‘Qiagen DNeasy blood and tissue’ kit) and PCR (see Sections 4.2.2 and 4.2.5 for extraction and PCR procedures), to assess whether carryover is likely to occur without any precautionary measures.

2. Using a separate set of frog tissue samples (n = 5), a similar protocol was followed, but the decontamination steps outlined above were applied before running each probe
in 3 ml DNase-free water, to ensure the protocol did indeed remove carryover contamination.

3. A final set of frog tissue samples (n = 5) were subjected to identical protocol as those in step 1, but after running the probe in 3 ml DNase-free water the probe was subjected to the decontamination steps and rerun in the water, to assess whether any potential carryover of bleach may have negatively affected DNA detection.

4.2.2 DNA extraction

An initial pilot study was carried out to compare two DNA extraction techniques on stomach contents: Chelex extraction (as described in section 4.2.3) and 'Qiagen DNeasy blood and tissue' kit extraction (Qiagen; following the manufacturer’s instructions). This kit has been used for previous molecular diet studies (Kvitrud et al. 2005; Vestheim and Jarman 2008; Cassel-Lundhagen et al. 2009; Braley et al. 2010; Heidemann et al. 2011; Weber and Lundgren 2011). Stomach samples from laboratory Norway rats (n = 28) and hedgehogs (n = 14) were divided into two, with one half being used in Chelex extraction and one half being used in Qiagen kit extraction. The results presented in section 4.3.4, show that Qiagen extraction outperformed Chelex extraction and hence all further stomach samples were extracted using the Qiagen kit.

DNA from faecal samples was extracted using the 'Zymo D6010 Faecal DNA' kit (Zymo Research) following the manufacturer's instructions. This was shown to outperform other faecal extraction kits in a number of studies (Yoshikawa et al. 2011; Jedlicka et al. 2013; Leite et al. 2013).

4.2.3 Primer design

Mitochondrial DNA (mtDNA) was chosen as a target on which to base primers, because differences in mtDNA among animal species are large (Kocher et al. 1989). Also, the
presence of a high copy number of mtDNA in each cell significantly increases the sensitivity of PCR assay (Girish et al. 2004; King et al. 2008). Within mtDNA, the 12S gene was chosen as a target for amplification. Although the rate of evolution of the mitochondrial genome exceeds that of the nuclear genome by a factor of about 10 (Brown et al. 1979), 12S is a relatively slowly evolving gene within the mtDNA genome (Pesole et al. 1999). This characteristic results in little within-species and large among-species variation, making it an ideal unit for distinguishing between species (King et al. 2008) and it has previously been targeted in molecular analyses of predation (Sutherland 2000; Dodd 2004; Jarman et al. 2004; Harper et al. 2005; Harper et al. 2006; Jarman et al. 2006; Passmore et al. 2006).

To design primers targeting the 12S gene of three of the anuran species present on mainland New Zealand, 12S sequences of all mainland anuran species were required. Sequences were available on the Genbank® database (http://www.ncbi.nlm.nih.gov/) for green and golden bell frogs (Litoria aurea; Accession no: AY819398), Hochstetter’s frog (Accession no: DQ28321) and Archey’s frog (Accession no: DQ283216). It was necessary to partially sequence the 12S gene for the remaining two mainland species (southern bell frog and brown tree frog Litoria ewingii). Tissue from both of these species was subjected to DNA extraction using a Chelex method: tissue was lysed using 200 µg proteinase K in 400 µl 5% Chelex 100 solution (Sigma-Aldrich; Walsh et al. 1991) and DNA was purified using standard ethanol precipitation (Dieffenbach and Dveksler 2003). Amplification of 12S was carried out using a universal 12S primer set (L1091/H1478; Kocher et al. 1989) with the following PCR conditions: 10 µl reactions containing 30 ng DNA, 1 x NH₄ buffer (BIOLINE), 1.5 mM MgCl₂ (BIOLINE), 0.2 mM dNTPs, 0.5 µM of each primer (forward and reverse), 0.5 U BIOTAQ (BIOLINE). The thermal cycling profile used a Touchdown PCR, as this favours the most specific primer-template interactions (Korbie and Mattick 2008): initial step for 2 min at 94 °C; then 10 cycles of the following: denaturation at 94 °C for 20 s, annealing at varying temperatures for 25 s (60 °C for initial cycle, then decreasing by 1 °C for each further
cycle), and extension at 72 °C for 1 min; then 25 cycles of the following: denaturation at 94 °C for 20 s, annealing at 50 °C for 25 s, and extension at 72 °C for 1 min (using the Eppendorf Mastercycler Pro 6321 PCR machine). The PCR products were then sequenced using the ABI 3730xl DNA Analyser (Applied Biosystems). Sequences are provided in Appendix H.

Using the online application Primer-BLAST, hosted by the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/tools/primer-blast), potential primers were generated for the 12S gene of each the target species (Archey’s frog, Hochstetter’s frog and southern bell frog). Primer-BLAST attempts to find primers specific to the input template sequence by testing the generated primers against the GenBank® ‘nucleotide’ database, to ensure that no other, non-target, sequences will be amplified. A review carried out by King et al. (2008) suggested that primers for use in prey DNA amplification should have high melting temperatures, as this allows PCRs to run with high annealing temperatures, reducing the risk of non-specific amplification. Targeted DNA fragments should also be short (c. 100 – 300 bp), as DNA is broken into smaller fragments during digestion (King et al. 2008). These characteristics were specified during the input stage of the Primer-BLAST searches. Based on the Primer-BLAST output, 8 primer sets were chosen for trial on New Zealand mainland frog species’ DNA. It should be noted that according to Primer-BLAST, it was not possible to develop a primer specific to the 12S sequence of southern bell frogs. All potential southern bell frog primers would apparently amplify green and golden bell frog (L. aurea) also.

4.2.4 Optimising primer performance

Tissue samples of all anuran species with naturally occurring populations on mainland New Zealand (brown tree frog, southern bell frog, green and golden bell frog, Archey’s frog and Hochstetter’s frog) were obtained from collections held by the University of Otago. Along with these samples, the liver tissue of a laboratory Norway rat, a ship rat, a mouse and a hedgehog, was subjected to DNA extraction using the Chelex/ethanol precipitation method
Chapter 4 – Detecting frogs as prey using DNA-based analysis

outlined in Section 4.2.3. DNA concentration was measured by mass spectrophotometry (Thermo Scientific NanoDrop™ 1000 Spectrophotometer) and, as DNA concentration was found to vary considerably, samples were diluted to c. 30-50 ng µl.

Using the newly designed primers, initial PCRs were performed on all the anuran and mammalian DNA, testing each primer pair. Initial PCR procedure was as follows: 10 µl reactions containing 30 ng DNA, 1 x NH₄ buffer (BIOLINE), 1.5 mM MgCl₂ (BIOLINE), 0.2 mM dNTPs, 0.5 µM of each primer (forward and reverse), 0.5 U BIOTAQ (BIOLINE). The thermal cycling profile was: initial step for 2 min at 94 °C; then 30 cycles of the following: denaturation at 94 °C for 15 s, annealing at 45 °C for 25 s, and extension at 72 °C for 30 s (using the Eppendorf Mastercycler Pro 6321 PCR machine).

PCR products were size fractionated on a 2 % agarose gel (BIOLINE agarose) containing 2 X fluorescent nucleic acid stain (SYBR safe DNA gel stain, Invitrogen) in 1 X TAE (40mM Tris, 1 mM EDTA, 20 mM acetic acid, and 1 mM EDTA, pH 8.0) at 100 V for 30 min.

This confirmed that all primers amplified their respective target taxon, regardless of whether they also amplified non-target taxa. Next, a series of similar PCRs were carried out increasing the annealing temperature in increments of 3 °C (initially 45 °C), to identify the temperature at which PCRs amplified only their respective target taxon’s DNA. Finally, another series of PCRs were run in which MgCl₂ concentrations were steadily increased in increments of 0.5 mM (initially 1.5 mM). MgCl₂ can affect the sensitivity and specificity of PCR (King et al. 2008) and, to allow for maximum sensitivity, the highest levels of MgCl₂ possible, while ensuring species-specificity of each primer, were used for all PCRs from this point.

Based on the results, one primer pair for each of the three target species was chosen (Table 4.2). The final PCR procedure for those three primer pairs was as follows: 10.5 µl reactions
containing 30-50 ng DNA, 1 x NH$_4$ buffer (BIOLINE), 3.8 mM MgCl$_2$ (BIOLINE), 0.2 mM dNTPs, 0.5 µM of each primer (forward and reverse), 0.5 U BIOTAQ (BIOLINE). The thermal cycling profile was: initial step for 2 min at 94 °C; then 35 cycles of the following: denaturation at 94 °C for 15 s, annealing at 69 °C for 25 s, and extension at 72 °C for 30 s (using the Eppendorf Mastercycler Pro 6321 PCR machine).

4.2.5 PCR procedure for stomach and faecal samples

Once stomach and faecal samples had been collected, homogenised and subject to DNA-extraction, they were ready for PCR. All PCRs included positive controls (PCR reagents with southern bell frog DNA) to confirm suitable reaction conditions, and negative controls (PCR master mix without any DNA) to check for contamination. Aerosol-resistant pipette tips (Axygen Scientific, Inc.) were used throughout all PCR procedures. Initial positive samples were also sequenced and aligned with known southern bell frog 12S sequences to ensure the amplified products did belong to this species, rather than that of predator DNA. Where it was apparent that a PCR had been inhibited by a DNA concentration that was too high (by the presence of excessive visible stained DNA along the entire length of the agarose gel), the concentration was measured, samples diluted to c. 30 - 50 ngµl and the PCR rerun. Also, a small tissue sample from each frog fed to a subject was first subjected to PCR, confirming that prey DNA could be amplified in all cases prior to prey ingestion.

Following Murphy et al. (2003), a second PCR was carried out on any samples that did not amplify during the first attempt so as to minimize the impact of stochastic pipetting error. Kvitrud et al. (2005) also found that multiple PCRs increased prey detection, and concluded that this was most likely due to differences in the DNA template for each PCR run, with prey DNA in some samples being highly degraded and present in very small quantities.
4.2.6 Statistical analysis

All statistical analyses were carried out using SPSS Version 20.0 (IBM Corporation, IBM SPSS Statistics for Windows, New York, U.S.A.), unless otherwise stated. Differences in PCR detection rates between Chelex and Qiagen kit extraction were evaluated by chi-squared contingency tables.

Probit regression was performed on the detection rate data of southern bell frog prey as identified by PCR and used to estimate the half-life of prey detection (hours at which probability of detecting prey = 0.5). Probit regression is often used to estimate the median lethal dose (the dose which has been found to be lethal to 50 % of test subjects) of a compound (Williams 1986; Takeuchi and Endo 2012; Sáez et al. 2013). Similarly to lethal dose, prey detectability has been found to be exponential in numerous studies and probit regression is considered to be the appropriate analysis for this type of data (Chen et al. 2000; Payton et al. 2003; Greenstone et al. 2007; Greenstone et al. 2010). SAS System for Windows Version 9.0 (SAS Institute Inc., Cary, NC, USA) was used to run probit regressions using the PROC PROBIT function. The absence of an overlap of 84 % fiducial limits around the predicted half-life indicates significance between groups at the $\alpha = 0.05$ level (Payton et al. 2003; Greenstone et al. 2007; Greenstone et al. 2010). Two regressions were performed, one for stomach content data and one for faecal content data, with each regression including all species groups (as a factor – groups listed in Table 4.1) and all time intervals (as a covariate). A theoretical time interval of 0 h was included for each mammal species, where probability of detecting prey at 0 h = 1.0. This assumes that prior to frogs being subjected to ingestion, DNA can be successfully amplified from those frogs in 100 % of cases. Because DNA was amplified from each frog prior to predators ingesting them, this appeared to be a suitable assumption.
4.3 Results

4.3.1 Homogeniser carryover experiment

Southern bell frog DNA was detectable in all water samples \((n = 5)\), indicating that carryover contamination between samples is very likely in the absence of a decontamination protocol. With the decontamination steps included in the protocol none of the water samples \((n = 5)\) contained amplifiable DNA, demonstrating that the decontamination with bleach was effective. All PCRs were run twice, so the lack of positives indicated that any DNA, if present, was not amplifiable even given differing templates available in each PCR. Where probes had been subject to decontamination steps prior to running in contaminated water samples \((n = 5)\), all samples tested positive for southern bell frog DNA, indicating that the rinsing steps sufficiently removed bleach from the probes.

4.3.2 Hedgehog washing

Of the four final water baths collected, one tested positive for southern bell frog DNA. This was a very weak amplification, as judged by the intensity of the band viewed on the transilluminator. Because this was a worst case scenario, using 2 g of pulverised frog tissue, and as no southern bell frog DNA was detected in three out of the four washing trials, this protocol was considered adequate. Residual faeces would contain far less DNA, it would be highly degraded, and it would contain PCR inhibitors (reviewed in King et al. 2008), therefore the likelihood of prey DNA carryover during actual faecal collection trials would be far less.
Table 4.2 Final primer pairs, associated product sizes (base pairs, bp) and annealing temperatures used in this study.

<table>
<thead>
<tr>
<th>Target species</th>
<th>Forward primer 5’-3’</th>
<th>Reverse Primer 5’-3’</th>
<th>Product size (bp)</th>
<th>Annealing T (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Leiopelma archeyi</em></td>
<td>12S-LA-F</td>
<td>12S-LA-R</td>
<td>129</td>
<td>69</td>
</tr>
<tr>
<td></td>
<td>GGCTGGTATCAGGCACATA</td>
<td>CCGGCTCTGGTAGCTGTAA</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Leiopelma hochstetteri</em></td>
<td>12S-LH-F</td>
<td>12S-LH-R</td>
<td>172</td>
<td>69</td>
</tr>
<tr>
<td></td>
<td>AACACTAGCCAAGCGGCTGT</td>
<td>TTCCCTGGCGGGATGTGAA</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Litoria raniformis</em></td>
<td>12S-LR-F</td>
<td>12S-LR-R</td>
<td>130</td>
<td>69</td>
</tr>
<tr>
<td></td>
<td>GCTTAATGTCCAAACGTCAT</td>
<td>GCTAAATCCCGCTTCTAATAG</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
4.3.3 Primer specificity

DNA from all mainland frog species was extracted successfully and clearly amplifiable using the universal 12S primers (Kocher et al. 1989). Once primer optimisation had been completed, specificity was confirmed for the three primer pairs designed in this study (e.g. Figure 4.1). Contrary to the results of Primer-BLAST, it was possible to attain species-specificity using the output primers for southern bell frogs, but only by increasing the annealing temperature to a high level (69 °C).

![Figure 4.1 Example of specificity of primer pair 12S-LR-F/12S-LR-R, targeting southern bell frogs. Band (indicated by arrow) indicates successful amplification of southern bell frog DNA, while absence of bands indicates that DNA from non-target taxa was not amplified. Dark bands indicate the loading dye used (xylene cynol).](image)

4.3.4 Chelex versus Qiagen kit DNA extraction

Based on the number of successful DNA amplifications following PCR, DNA extraction using the Qiagen kit was substantially more efficient than Chelex extraction for stomach samples (Table 4.3). The number of amplifications did not differ significantly between methods where rats had ingested entire frogs, but based on the large $\chi^2$ value obtained for other group comparisons, only the Qiagen kit was used for stomach sample extractions from this point on.
Table 4.3 Frequency of positive DNA amplifications of southern bell frog DNA from laboratory Norway rat and hedgehog stomach samples, following Chelex and Qiagen kit extraction methods.

<table>
<thead>
<tr>
<th>Group</th>
<th>Chelex</th>
<th>Qiagen kit</th>
<th>( \chi^2 ) (df, n), p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>laboratory Norway rats (consumed entire frogs)</td>
<td>13 %</td>
<td>62.5 %</td>
<td>( \chi^2 ) (1,8) = 4.27, ( p &gt; 0.15 )</td>
</tr>
<tr>
<td>laboratory Norway rats, (consumed frog muscle)</td>
<td>0 %</td>
<td>75 %</td>
<td>( \chi^2 ) (1,20) = 24.0, ( p &lt; 0.001 )</td>
</tr>
<tr>
<td>hedgehogs (consumed entire frogs)</td>
<td>36 %</td>
<td>86 %</td>
<td>( \chi^2 ) (1,14) = 7.34, ( p &lt; 0.01 )</td>
</tr>
<tr>
<td>total</td>
<td>14 %</td>
<td>76 %</td>
<td>( \chi^2 ) (1,42) = 32.49, ( p &lt; 0.001 )</td>
</tr>
</tbody>
</table>

**4.3.5 Detectability of prey DNA**

It was possible to amplify and detect southern bell frog DNA from small mammal stomach and faecal contents using the protocols developed in this study. Sequences from successfully amplified prey DNA were most similar to that of southern bell frog, rather than that of other mainland frog species or predator subjects (Appendix I). None of the control samples of stomach or faecal contents, from animals that had not ingested frog material, tested positive for the presence of southern bell frog DNA, indicating there was not, at least, a systematic contamination of samples taking place throughout any of the procedures. The probit regression models fitted the data well for both stomach contents \( \left( \chi^2 \right)_{18, \text{n} = 95} = 16.67, \ p > 0.55 \) and faecal contents \( \left( \chi^2 \right)_{22, \text{n} = 199} = 24.41, \ p > 0.3 \) and the slopes of prey detection rate were significantly different from zero for all predator species groups \( (p < 0.001 \text{ for all groups}) \) showing that the detection rate did indeed decay over time. The probability of detecting frogs as prey following prey ingestion is shown for all subject groups in Figures 4.2 and 4.3. For comparison, the half-lives of prey detection among predator species are plotted in Figure 4.4, with groups clustered according to absence of significant differences, based on the overlap of 84 % fiducial limits.
On average, using faecal samples rather than stomach samples increases the prey detection half-life 3.17-fold, from 6.74 h to 20.85 h (see Figure 4.4). However, observed prey detection probability peaked at an average of 1.0 for stomach contents 1 h following prey ingestion, whereas it only peaked at an average 0.74 for initial faeces collections (which included all faeces produced up to that point; Figures 4.2 and 4.3).

Compared to using traditional visual identification, DNA-based analysis greatly improved prey detection probabilities (Table 4.4). Determining the half-life of detecting frogs as prey (to order level) in rodent faeces using traditional morphological analysis is not possible, and in stomach samples it was less than 1 h (Chapter 3). DNA-based methods extend this half-life to c. 6 h (stomach contents) and c. 19 h (faeces). In hedgehogs, the half-life was extended from 1.77 h to 9.16 h and 10.73 h to 29.15 h for stomach and faecal contents respectively.

**Table 4.4** Proportion (%) of stomach (n = 95) and faecal (n = 199) samples in which frogs as prey were identified using morphological (to order level) or DNA-based analysis (to species level). LNR – laboratory Norway rat. Total % does not include data from the ‘LNR (muscle ingested only)’ group (stomach n = 23, faeces n = 36) as these samples were not subject to morphological analysis.

<table>
<thead>
<tr>
<th>Species</th>
<th>Morphological analysis</th>
<th>DNA-based analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% stomach</td>
<td>% faeces</td>
</tr>
<tr>
<td>LNR</td>
<td>5.56</td>
<td>0</td>
</tr>
<tr>
<td>LNR (muscle ingested only)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Ship rat</td>
<td>6.67</td>
<td>0</td>
</tr>
<tr>
<td>Mouse</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Hedgehog</td>
<td>20.8</td>
<td>36.73</td>
</tr>
<tr>
<td>Total (n^{stomach} = 72, n^{faeces} = 163)</td>
<td>9.72</td>
<td>11.04</td>
</tr>
</tbody>
</table>
Figure 4.2 Probability of detecting southern bell frogs as prey in wild mouse, wild ship rat, and wild hedgehog stomach (solid line) and faecal (double line) contents, using DNA-based methods. Dotted and dashed lines indicate 95% fiducial limits for stomach and faecal contents respectively. Also shown are observed proportions of positives for stomach (■) and faecal samples (●).
Figure 4.3 Probability of detecting southern bell frogs as prey in laboratory Norway rat stomach (solid line) and faecal (double line) contents, using DNA-based methods, where rats had consumed either entire frogs, frog flesh only, or the data from both groups has been combined. Dotted and dashed lines indicate 95% fiducial limits for stomach and faecal contents respectively. Also shown are observed proportions of positives for stomach (■) and faecal samples (●).
Figure 4.4 The half-lives (h) for the detection of southern bell frogs as prey in small mammal stomach and faecal contents, using DNA-based methods. LNR - laboratory Norway rat; EF - consumed entire frogs; FF - consumed frog flesh only; combined - combined data for EF and FF. Umbrella brackets indicate predator species groups that are not significantly different at $\alpha = .05$. Error bars indicate 84% fiducial limits.
4.4 Discussion

The investigation of prey DNA detection rates over time has often been reported for invertebrates predating on invertebrate prey (e.g. Asahida et al. 1997; Agustí et al. 1999; Agustí et al. 2000; Chen et al. 2000; Symondson 2002 [review]; Greenstone et al. 2007; Weber and Lundgren 2009; Greenstone et al. 2010). Such data have not been collected to the same extent for vertebrates, but have been reported for Steller sea lion faeces (*Eumetopias jubatus*; up to 48 h prey detection; Deagle et al., 2005b), seal faeces (*Arctocephalus* spp.; 95% of prey detected within 39.5 h; Casper et al., 2007), mackerel GIT (*Scomber scombrus*; up to 12 h prey detection; Rosel and Kocher, 2002), whiting stomachs (*Merlangius merlangus*; prey detection half-life = c. 31 h; Hunter et al., 2012), sunfish (*Lepomis* spp.) and rockbass (*Ambloplites rupestris*) stomachs (prey detection half-life = c. 10-12 h; Carreon-Martinez et al., 2011) and crow faeces (*Corvus corone corone*; up to 4 h prey detection; Oehm et al., 2011). This is the first study to examine prey DNA detectability over time in mammal stomach contents, and the first to compare stomach versus faecal prey DNA detectability over time in any vertebrate. Such data will be very useful when designing and analysing results of field studies investigating small mammal diets (see Chapter 5 for an example of this), and can also be used to adjust raw incidence-of-prey data to rank predator impacts (Greenstone et al. 2007; Greenstone et al. 2010).

The results presented in this chapter indicate that it should be possible to estimate the impact of small mammals on amphibian prey species using kill-trapping methods, followed by DNA-based stomach content analysis. This is because prey in stomach contents will only be detectable for up to one night (the mammal species included in this study all being nocturnal). If prey is detected in the stomach contents of a kill-trapped small mammal, it is certain that the predator consumed the prey item during the night it was trapped, therefore the predation rate can be estimated based on the number of predation events observed per night. This is
more complicated using small mammal faeces as there is potential to detect prey in faeces collected on the second night following the predation event. In this case a comparative weighting can be applied on prey positives so that predation is not overestimated on a given night or between different predators (King et al. 2008).

Using the methods described herein only the minimum number of prey consumed per sample could be estimated, as the technique does not distinguish between cases where a predator has consumed one prey item or many. Zaidi (1999) showed that number of prey eaten had no effect on prey DNA detectability. Using quantitative PCR, other researchers have linked the amplicon quantity to the number of prey items consumed (Weber and Lundgren 2009) and have been able to approximate the relative quantity of prey (Bowles et al. 2011). However, such quantitative prey estimate protocols are known to pose problems, particularly a bias in favour of hard to digest prey, when multiple prey species have been ingested in unequal amounts (Deagle et al. 2005b; Deagle and Tollit 2007) or when prey species differ in amount of DNA present per unit biomass (Deagle et al. 2010). Pooling of samples, DNA purification, PCR amplification and DNA sequencing could also be influencing factors (Deagle et al. 2010).

A recent study by Zarzoso-Lacoste et al. (2013) included analysis of prey DNA from the stomach and faecal contents of wild kill-trapped ship rats (n = 5) and pacific rats (n = 7; *Rattus exulans*), using three group-specific primer sets (targeting birds, invertebrates and plants), followed by cloning of PCR products in order to sequence amplicons that contained DNA from multiple taxa within a target group. Their study did not include analysis of samples over time but was a snapshot of predator diet, identifying 16 prey species in the rats’ diets (n = 5; randomly selected samples). In contrast to the present study, they found that the DNA-based approach yielded similar results to morphological techniques. This is likely to be highly affected by the prey type being investigated. For example, as noted by Zarzoso-
Lacoste et al. (2013), chitinous and keratinous material (such as that found in arthropod exoskeletons, bird feathers and animal scales or hairs) survives digestion very well, allowing morphological detection, but contains a relatively low amount of DNA (Symondson 2002), hampering DNA-based detection. It is apparent from this thesis that the same is not true when small mammals prey on frogs, as diagnostic frog material may not even be ingested (Chapter 2) or, if it is ingested, it does not survive ingestion particularly well (Chapter 3), but it does contain DNA at levels suitable for detection.

The DNA-based methods used in the present study have greatly extended the half-life of prey detectability in small mammals in comparison to traditional morphological techniques (Chapter 3). In the majority of cases the observed probability of prey detection at a given time interval was similar to, or less than, that observed during the preceding time interval. This is to be expected as, for example, prey DNA becomes degraded during digestion (Harper et al. 2005; Deagle et al. 2006), or as prey move from the stomach to the small intestine, leading to a lower rate of DNA detection.

However, in a few cases the observed results did not conform to those predicted (e.g. there was a higher proportion of samples positive for prey in mouse stomach contents at 6 h than at 3 h, and a higher proportion positive for hedgehog stomach contents at 9 h than at 6 h). These anomalies may be an artifact of false positives (i.e. contamination with prey DNA), false negatives (the insensitivity of the developed protocol to detect prey DNA consistently) and/or small sample sizes (in the cases of stomach contents). It is possible that cross contamination of samples during PCR may have occurred, but every eight PCRs included a negative control (only reagents) and the lack of amplification in these sample wells indicates that a systematic contamination was not occurring. A second PCR run often yielded a positive reaction where the first had not, indicating that there is some stochasticity involved. This was also noted by Murphy et al. (2003) and Kvitrud et al. (2005) who concluded it was probably due to
differences in the DNA template for each run. For some individuals, faecal time series also yielded unexpected results, for example, the faeces for one mouse was positive for frogs as prey at 6 h, followed by a negative result at 12 h, followed by another positive at 18 h. The only apparent explanation here is the production of a false negative at 12 h, rather than any particular degradative effect on prey DNA in the GIT during the 6 – 12 h faeces collection interval. A partitioning of GIT transit that would cause prey to be defecated once at 6 h, and again at 18 h, but not at 12 h is also considered unlikely. Therefore, in some cases it appears that differences in DNA template, affecting PCR reliability, and perhaps relatively small sample sizes (for stomach contents) are the primary factors influencing these anomalies, although differences in the time that samples were kept in storage may also have played a role (see Section 4.4.1.1 for more on this).

There was no significant difference in prey detection where laboratory Norway rats had been fed either entire frogs or only frog soft tissue, indicating that the presence of skeletal material in the predator stomach and faecal contents did not greatly influence the DNA detection rate. Even in the case of mice, which only consumed 0.1 g of frog soft tissue, prey DNA was often recoverable (67 % of stomach samples, 43 % of faecal samples).

In all cases, the half-life detection of prey DNA equalled or exceeded known gastric emptying or GIT transit times (Table 4.5). The exception to this may be prey detection in ship rat faeces, which had a relatively short half-life of 12.89 h, but the GIT transit for this species is unknown. Stomach emptying in small mammals is often reported in terms of a half-life, but it should be noted that the half-life of gastric emptying (time at which half of the ingested meal has passed into the small intestine) is a very different measure to the half-life of prey detectability reported in the present study.
Table 4.5 Reported gastric emptying times and total gastrointestinal (GIT) transit times (h) alongside prey DNA detection half-lives (present study) for the small mammal species included in this study.

<table>
<thead>
<tr>
<th>Species</th>
<th>Gastric emptying</th>
<th>Prey DNA detection half-life: stomach contents</th>
<th>Total GIT transit</th>
<th>Prey DNA detection half-life: faecal contents</th>
</tr>
</thead>
<tbody>
<tr>
<td>mouse</td>
<td>0.5 h (half-life)</td>
<td>5.3 h</td>
<td>10.6 h</td>
<td>15.8 h</td>
</tr>
<tr>
<td>laboratory Norway rat</td>
<td>1.6 h (half-life)</td>
<td>5.2 – 6.8 h</td>
<td>15 – 22 h</td>
<td>22 - 24 h</td>
</tr>
<tr>
<td>ship rat</td>
<td>Complete emptying took over 6 h</td>
<td>7.26</td>
<td>Not reported in literature</td>
<td>12.89 h</td>
</tr>
<tr>
<td>hedgehog</td>
<td>Not reported in literature</td>
<td>9.16</td>
<td>6 – 12 h, 12-16 h</td>
<td>29.15 h</td>
</tr>
</tbody>
</table>

Osinski et al. (2002); Bellier et al. (2005); Dropleman et al. (1980), Enck et al. (1989); Sigleo et al. (1984) and Enck et al. (1989); Weld et al. (2004); present study (Chapter 3); Reeve (1994)

Overall, it appears that it is the passage of prey through the GIT that limits the successful detection of DNA, rather than the effects of DNA degradation while still in the GIT. Therefore, it is expected that further attempts to increase the sensitivity of the developed protocol, or decrease the potential effects of inhibition, would lead to only marginally longer prey detection times. This is a major improvement over the half-life detection when using morphological identification methods (Chapter 3). Nonetheless, it is valuable to explore factors that may influence prey detection rates and these are discussed in Section 4.4.1.

4.4.1 Factors potentially influencing the observed prey detection rates

4.4.1.1 Sample storage

There have been many different approaches to stomach and faecal sample storage (Frantzen et al. 1998; reviewed in King et al. 2008) including freezing, ethanol, ethanol plus freezing, silica gel, commercial kits, and dimethyl sulfoxide salt solution. A number of studies have reported success using 95 % ethanol followed by storage at room temperature (Deagle et al. 2005a; Deagle et al. 2005b; Deagle et al. 2009; Deagle et al. 2010) and this was followed in the present study. This technique was chosen because it is a relatively easy method to use.
when collecting field samples, and DNA was successfully amplified up to 28 months after collection. However, as some samples were kept in storage for much longer periods than others, this may help to explain the anomalous results referred to in Section 4.4.

4.4.1.2 Homogenisation and DNA extraction

Deagle et al. (2005b) found that blending faecal samples increased the prey detection rate for sea lions fed a known diet, when compared to faecal subsampling. The homogenisation protocol carried out in the present study was shown to be efficacious in minimising contamination between samples and did not cause degradation of DNA due to potential carryover of bleach.

The Qiagen DNeasy blood and tissue kit extraction far outperformed Chelex extraction. There are two primary potential causes for this outcome: 1) Chelex extraction was not as efficient as the Qiagen DNeasy kit at extracting DNA or; 2) Chelex extraction resulted in the co-precipitation of unwanted PCR inhibitors. The former possibility is deemed unlikely as spectrophotometry revealed that Chelex-extracted samples generally contained a much higher concentration of DNA than those extracted using the Qiagen DNeasy kit. The latter possibility is much more likely as the absorbance ratio of UV-260:280 indicated that Chelex-extracted DNA samples were not as pure (data not shown). There are a number of known PCR inhibitors, including bile salts (Lantz et al. 1997), calcium and collagen (Opel et al. 2010) and it is likely that these co-precipitated to a greater degree using the Chelex method. Other molecular predator diet studies have used a variety of extraction techniques including phenol / chloroform (Rosel and Kocher 2002; Sheppard et al. 2004) and cetyltrimethyl ammonium bromide (Agustí et al. 1999; Agustí et al. 2000; Juen and Traugott 2005). However, commercial kits are being used ever more prevalently (Agustí et al. 2003; Kasper et al. 2004; Foltan et al. 2005; Harper et al. 2005; Casper et al. 2007b; Deagle and Tollit 2007), as they are often easy to use and can prove effective at overcoming the problems
of co-extracted PCR inhibitors (King et al. 2008). There are many such kits available and it is likely that different kits will yield differing results. The Qiagen DNeasy kit appeared to work very well for stomach samples in the present study and was shown to be the most efficient of 8 different prey DNA extraction protocols in a study by Simonelli et al. (2009) investigating invertebrate diet. The Zymo kit worked well for faecal samples and has also been used successfully (usually outperforming other brands) in previous faecal DNA studies (Yoshikawa et al. 2011; Jedlicka et al. 2013; Leite et al. 2013).

4.4.1.3 PCR

Alterations to the PCR protocol could result in a higher rate of detectability. One such alteration could be the addition of PCR facilitators. Juen and Traugott (2006) found the addition of bovine serum albumen (BSA) to greatly increase prey DNA amplifiability. Simply diluting the extract prior to PCR is another approach to overcome PCR-inhibition, however, Juen and Traugott (2006) do not recommend this approach when dealing with prey DNA as the prey DNA concentration is likely to be much lower than the overall DNA content and a critical factor after several hours of digestion.

Different primers have also been shown to result in different half-lives even within prey species (Juen and Traugott 2005), mainly because they target fragments of different sizes, which will survive digestion at varying rates. It is therefore possible that the primers developed for Leiopelma spp. in this study may exhibit different detectability rates than that examined for southern bell frogs. However, the sizes of the targeted fragments are similar for the three primers (Table 4.2) and the half-life of these small prey DNA fragments appears to be dictated by the passage of prey through the GIT rather than DNA degradation while still in the GIT. One possible way to overcome any differences in prey detection rate, caused by using different primers for each prey species, is to use group-specific primers. A collaborative project, running alongside the research presented in this thesis, aims to develop
and test group specific primers that target all frog species as prey. This has largely been successful and the primers have been used to identify frogs as prey in field-collected small mammal stomach samples (see Appendix J for details of the results). One problem with these primers is ensuring that DNA from the majority of frog species worldwide will be amplified, while DNA from non-target prey species and from the respective predator species will not. So far, the developed primers and associated PCR protocol amplify the DNA of c. 90% of the frog species tested (57 species from all major frog families). However, non-target species have also been amplified in some cases (a reptile species as prey, and mouse DNA as a predator). It is hoped that further research, including a refinement of the PCR protocols, will result in amplification of target species only.

The annealing temperature used for the primers in this study was relatively high (69 °C). This was necessary to ensure species-specificity, but lowering annealing temperatures has been shown to result in a marked increase in prey DNA detection (Sint et al. 2011).

Altering PCR protocols may help to elucidate the reasons behind the anomalous results referred to in Section 4.4.

4.4.1.4 Meal composition

Meal composition influences the secretion of gastric acid, digestive enzymes and gut hormones in mammals (Jobling 1986). The mammalian subjects in this study were only fed dry food pellets prior to and post frog prey ingestion. This standardised the results, but is not analogous to field conditions, as predators may have consumed alternative prey items prior to and following frog ingestion. The ingestion of alternative prey following target prey has been shown to extend prey DNA detection times in invertebrates (Dodd 2004). All subjects in the present study were allowed ad libitum access to dry pellets following frog ingestion, therefore detection rates observed in this study may be longer than those in similar field studies.

Calcium from bone material is often co-extracted and is a PCR inhibitor (Eilert and Foran
but adding MgCl$_2$ up to three times the normal concentration has been shown to increase amplification success in the presence of calcium (Opel et al. 2010). While the present study did not empirically investigate the effects of bone, the PCR optimisation steps (including increased MgCl$_2$ concentrations) resulted in a higher success rate for prey DNA amplification.

### 4.4.2 Conclusions

This is the first study to examine prey DNA detectability over time in mammal stomach contents, the first to compare stomach versus faecal prey DNA detectability over time in vertebrates and the first to focus on detecting amphibians as prey using DNA-based methods. The detection of prey equalled or exceeded known gastric emptying or GIT transit times, indicating that it is the rate at which prey passes from the stomach into the small intestine, or the rate at which it passes through the entire GIT that limits the successful detection of DNA, rather than the effects of DNA degradation while still in the GIT. It has resulted in a protocol for identifying frogs as prey in small mammal stomach and faecal samples that is much more reliable than traditional morphological analysis. The species-specific primers developed in this study can be used to carry out field-based diet analyses in small mammals to detect predation on their target anuran species. The protocol can easily be extended to other prey species through the design of relevant species-specific primers. However, a number of factors must be taken into account when analysing the results of such a study, including prey detection rates over time, differences in amplicon detection rates due to the application of different primers, potential for PCR inhibition, the effects of different storage and DNA extraction methods, and the predator and prey species involved.
Chapter 5 Estimating the predation rate of small mammals on frogs in New Zealand

Abstract

Molecular analysis of predation is a rapidly growing field used to study complex trophic interactions and detect the presence of endangered taxa in order to manage their conservation. The impacts of introduced mammals on New Zealand mainland native frog (*Leiopelma* spp.) populations are largely unknown and knowledge of those impacts are necessary to make informed decisions regarding the management of these endangered amphibian species. One species of introduced mammal often implicated in the decline of New Zealand frogs is the ship rat (*Rattus rattus*). The aim of this study was to utilise DNA-based diet analysis to provide estimates of the predation rate of small mammals, particularly ship rats, on frogs in New Zealand. Through the use of kill-trapping and bait tube placement, small mammal stomach and faecal samples were collected from three study areas in New Zealand known to have either native or introduced (*Litoria* spp.) frog species present. Ship rat densities were estimated in study areas where native frogs were known to be present. Stomach and faecal contents were subject to DNA-based analysis using species-specific DNA primers targeting three frog species (*Leiopelma archeyi*, *Leiopelma hochstetteri* and *Litoria raniformis*). A total of 5 samples (1.8%) tested positive for frogs as prey. Minimum predation rates of ship rats on native frogs were estimated at between 0.01 and 0.9 frogs/ha/night. It remains unclear whether native frogs can sustain this predation rate, but this is the first time New Zealand native frogs have been detected in introduced mammal stomach contents and the first time that DNA-based diet analysis has been used to determine the impacts of predators on amphibians. This provides a case study that can be followed by future conservationists researching the impacts of introduced fauna on native amphibians.
5.1 Introduction

A central goal in ecology is to understand the relationship between predators and prey, and the rate at which predators feed on prey is a major component of that relationship (Skalski and Gilliam 2001). For conservation purposes the quantification of ecological relationships, especially prey selection by predators, is a critical first step in the design of mitigation plans (Major et al. 2007). The impacts of introduced mammals on New Zealand mainland frog populations are largely unknown (see Chapter 1 of this thesis and Bishop et al. 2013) and in order to mitigate for the impacts of introduced predators on native frogs, we need first to know what those impacts are, or are likely to be. The main objectives of the research detailed in this chapter are to survey for predation by introduced mammals on frogs and to estimate the predation rate of ship rats (*Rattus rattus*) on native New Zealand frogs.

The evidence to date of introduced fauna negatively impacting *Leiopelma* is largely circumstantial. Notably, the extinction of three *Leiopelma* species occurred synchronously with the arrival of introduced fauna (in association with human settlers), as did the range contraction of the currently extant species (Worthy 1987b; Bell 1994b). All reports of predation events with frogs as prey in New Zealand are reviewed in Chapter 1 of this thesis. Prior to the present study there had been no research concentrating on the acquisition of direct evidence of introduced mammals depredating frogs in New Zealand (see Chapter 1 of this thesis and Bishop et al. 2013). The decline of *Leiopelma* spp. has often been attributed to the introduction of rodents (Towns and Daugherty 1994). Worthy (1987b) notes that the extinctions and range contractions are probably correlated with the arrival of the pacific rat (*Rattus exulans*), and that it is surprising that Archey’s frog (*Leiopelma archeyi*) and Hochstetter’s frog (*Leiopelma hochstetteri*) still exist at all on the mainland. Of the four rodent species present in New Zealand, ship rats appear to be the most numerous in New Zealand forests (Cunningham and Moors 1996; King et al. 1996), having superseded the
pacific rat (Atkinson 1973). As such, the potential impacts of ship rats on native frogs were given priority in this study.

In this chapter, I aim to survey for predation of frogs by introduced mammals in New Zealand, in order to extend the range of species known to be frog predators. I also intend to specifically estimate the predation rate of ship rats on the two remaining mainland native frog species (Archey’s frog and Hochstetter’s frog). To do so, I will use morphological and DNA-based diet analysis methods investigated and developed in Chapters 3 and 4 of this thesis. The development of the DNA-based methods used southern bell frogs (*Litoria raniformis*) as a model prey species (Chapter 3). Although southern bell frogs are not native to New Zealand, they are globally endangered and declining in their native Australian range (IUCN 2013), where Norway rats (*Rattus norvegicus*), ship rats and house mice (*Mus musculus*) have been introduced (Downes *et al.* 1997; Hocking and Driessen 2000). Information pertaining to predation on this species in New Zealand would therefore also be useful to conservationists in Australia. Furthermore, any positive amplifications of DNA of this species from field-collected mammal diet samples would provide a validation of those techniques.

5.2 Methods

5.2.1 Study areas

5.2.1.1 Whareorino Forest

Whareorino Forest is an extensive area of unlogged podocarp-hardwood forest (Pryde *et al.* 2006) situated in King Country, central North Island, New Zealand. The area is managed by the Department of Conservation (DOC) and is part of the Herangi Range, which extends from c. 400 m to c. 800 m above sea level (Haigh *et al.* 2007). The area is inhabited by Hochstetter’s frogs and a population of Archey’s frogs was discovered there in 1991 (Thurley and Bell 1994b; Thurley 1996), which appears to be in a stable condition (Ohmer *et al.* 2012).
Two study sites inhabited by both frog species were chosen based on the known presence of frogs from and advice from Lisa Daglish and Kate McKenzie (DOC, Maniapoto Area Office; pers. comm.; Figure 5.1).

5.2.1.2 The Waitakere Ranges
The Waitakere Ranges, situated west of Auckland, North Island, New Zealand, consist of a dissected plateau with an average elevation of about 340 m rising to 460 m in the highest region (Esler and Astridge 1974). Much of this area is covered by the Waitakere Ranges Regional Park which encompasses c. 16,000 ha of native forest and is administered by the Auckland Regional Council. The southern margins, forming part of the Manukau Entrance to Auckland Harbour, rise abruptly from the sea. Populations of Hochstetter’s frogs have been observed within and adjacent to riparian habitat across much of the area (Green and Tessier 1990; Tessier et al. 1991; Green 1994; Allen 2006; Moreno 2009; Nájera-Hillman et al. 2009a). Three study sites within the Ranges (Figure 5.1), known to possess Hochstetter’s frogs in high abundance, were chosen based on results from Moreno (2009).

5.2.1.3 Omakau
Initially, a field site in Alexandra had been proposed as a suitable site to carry out predator diet investigation for southern bell frogs as prey – a large number of frogs had been present there in previous years, with over 200 adults collected in a couple of nights in 2007 (Bishop 2008). However, during initial field surveys undertaken between November 2009 and January 2010, only 5 frogs were observed (the reason behind the low number of observations remains unknown). With prey at such low densities, predation events could be expected to be very rare, even if predators were actively hunting for frogs. The likelihood of detecting predation events would be far less again, and the absence of detection would not necessarily have yielded meaningful information. Therefore a different study area was sought. After considerable searching, and communication with many local people in the area of Dunedin and Alexandra, two sites in Omakau, Otago, New Zealand were found that had breeding
populations of southern bell frogs (Figure 5.1). Omakau is situated near to the Manuherikia River, and surrounded primarily by farmland at an elevation of roughly 350 m. Both study sites consisted of highly modified pastoral grassland (primarily utilised for sheep farming), divided by thin boundary hedgerows or fence lines. Southern bell frogs (adults and tadpoles) were generally confined to small drains and drain-fed farm ponds.

5.2.2 Trapping and stomach sample collection

In Whareorino Forest and the Waitakere Ranges, trapping consisted of a grid of 81 rat snap traps (Victor; Woodstream Corporation). In order to estimate the density of ship rats, the layout of the grid used the Zippin removal method (Zippin 1958) and was similar to that described by Brown et al. (1996). Initial trapping sessions consisted of 16 trap lines radiating from a centre point, each line was comprised of 5 rat snap traps, with a 25 m spacing between traps (plus an additional trap placed at the centre of the grid), following Brown et al. (1996). However, the results of these sessions (n = 2) indicated that the trap spacing was too far apart and that a low proportion of the rat population present was being trapped (see Section 5.3.2), which greatly affects the precision of the method (Zippin 1958). Consequently, for all other trapping sessions (n = 3) the spacing was decreased to 20 m (in a 200 m diameter grid; Figure 5.2). Rat traps were baited with peanut butter and placed under wire mesh tunnels with a plastic covering pegged into the ground, to reduce the risk to non-target species. Following Brown et al. (1996) the effective trapping area was calculated as 7.64 ha (the actual trapping area plus an additional radius of 56 m, based on the circular average home range of ship rats in mixed podocarp/broadleaf forest; Hooker and Innes 1995). For initial trapping sessions with 25 m trap spacing, the effective trapping area was 10.29 ha.

Additionally, five DOC 200 traps (DOC series trapping systems; Curtis Metal Products Ltd.), targeting mustelids, were positioned at 50 m intervals through the centre of the rat trapping grid. These were baited with minced rabbit meat (following Miller 2003; Moorhouse et al.
2003 and; Pierce \textit{et al.} 2007). All traps were left baited, but unset for the first night (following Hickson \textit{et al.} 1986; Tobin \textit{et al.} 1993). Traps were then set for 5 consecutive nights. Each morning, traps were checked, carcasses removed and traps reset if necessary. The status of each trap was also recorded (capture, bait missing, or sprung). Trap success was corrected for sprung traps (Nelson and Clark 1973).

To reduce the potential for environmental contamination of stomach samples, full predator carcasses were removed from the sites before being subjected to careful dissection using instruments washed in ethanol and flamed. Also, whole stomachs were taken and stored in 95\% ethanol, reducing the risk of contamination in the field. Stomachs were only emptied of their contents under laboratory conditions.
Chapter 5 – Predation on frogs in New Zealand

Figure 5.1 Locations of field sites and dates of fieldwork included in this study. Co-ordinates use the World Geodetic System 1984 (WGS1984) and are reported in decimal degrees. Maps and orthophotos sourced from Land Information New Zealand (LINZ) Data Service (http://www.linz.govt.nz/). Crown Copyright Reserved. This work is licensed under the Creative Commons Attribution 3.0 New Zealand License.
At the Omakau study sites, the habitat was not uniform, and mammalian fauna would not be expected to be distributed uniformly across the areas, rather utilising hedgerows, drains or fence lines (Pollard and Relton 1970; Tattersall et al. 2001; Gelling et al. 2007). Therefore, trapping was confined to these linear habitats along the margins of the pastoral grasslands, and around the pond areas where southern bell frogs were observed. Trapping was only carried out at Site 6 (see Figure 5.1). A total of 60 rat snap traps and 17 DOC 200 traps were employed for 5 nights. No attempt was made to estimate small mammal density at Omakau, but trap success was recorded.

Stomach contents of trapped animals were removed and stored in 95 % ethanol as this was found to be a suitable storage method for downstream DNA analyses (Chapter 4). The remains of the carcasses were buried on site. Rodent species were identified based on Cunningham and Moors (1996).

### 5.2.3 Faeces collection

To increase the number of small mammal diet samples collected per study site, bait tubes were used to collect faeces, similar to those developed by Churchfield et al. (2000) for water
shrews (*Neomys fodiens*) and subsequently used by Greenwood *et al.* (2002), Carter and Churchfield (2005), Poulton and Turner (2009) and Scott *et al.* (2012). Each bait tube consisted of a 220 mm length of plastic piping measuring 65 mm in diameter, covered at one end with opaque plastic covering and wire mesh (Figure 5.3). The bait tube design was then modified as follows: an internal compartment, inaccessible to visiting animals, was formed by cutting a slit in the piping and inserting a wall of wire mesh. Inside this compartment was placed a large bait (either peanut butter or rabbit meat; see Table 5.1). A very small bait was placed in the accessible portion of the bait tube. Bait tubes were held in place by pegging them on to the ground. The purposes of the modified bait tubes were: 1) to attract small mammals repeatedly over consecutive nights; 2) to entice visiting animals to stay in the immediate vicinity for long periods, thereby depositing multiple faecal pellets; 3) to only allow animals to obtain a very small amount of bait so that faecal samples would largely contain components representative of their usual diet.
At Whareorino Forest sites, where Archey’s frogs are found throughout the forest habitat, bait tubes were laid out similarly to rat snap traps (i.e. in lines radiating from a centre point), consisting of eight lines of 5 tubes each (n = 41 tubes: 8 lines of 5 plus one at centre point), with a 20 m spacing between tubes. Additionally, five tubes baited with rabbit meat, targeting mustelids, were positioned at 50 m intervals through the centre of the grid (Table 5.1).

At the Waitakere Ranges sites, because Hochstetter’s frogs are generally found very close to streams (McLennan 1985; Bell et al. 2004a; Moreno et al. 2011), and any small mammals consuming frogs would therefore also be close to the streams, tubes baited with peanut butter were positioned every 20 m (one on either side of the stream) for a stream reach of 300 m. Tubes baited with rabbit meat were placed every 50 m along the same reach (Table 5.1).

Again, because habitat was not uniform at Omakau study sites, bait tubes were confined to linear hedgerows, drains or fence lines, and around the pond areas where southern bell frogs were observed.

Among study sites, the number of nights that bait tubes were left out differed due to weather conditions and success rates (Table 5.1). The aim was to obtain faecal samples from nights
that frogs were active, so where prolonged periods of dry weather decreased frog activity to non-detectable levels (see Section 5.3.2, Figures 5.6 - 5.8), bait tubes were not checked.

Furthermore, no faecal samples were found in bait tubes at Omakau study sites and this field work was terminated after 14 nights.

All tubes were checked, and re-baited where necessary, each day. All faecal pellets found at any given bait tube were considered to be cross-contaminated, and as such were combined as one faecal sample. Disposable gloves were used to collect samples and were changed between each bait tube. Faecal samples were stored in 95% ethanol (see Chapter 4). For two nights in Whareorino Forest, a motion sensor camera (Bushnell Trophy XLT 119455) was installed to take photographs of animals visiting bait tubes.

Furthermore, any faeces observed on study sites, but not in bait tubes, were also collected opportunistically. Rodent faeces were identified based on recommendations by Cunningham and Moors (1996): mouse faeces 3.9 - 7.6 mm; ship rat faeces 6.8 - 13.8 mm; Norway rat faeces 13.4 - 19.1 mm.

5.2.4 Frog indices

All sites were searched at night at least once prior to placing bait tubes or traps to confirm the presence of frogs. To obtain an index of nightly frog emergence at each study area, a 50 m transect was walked each night during trapping and faeces collection. Transects were located near to (within 100 m), but not inside, trapping and bait tube grids at: 1) Whareorino Forest, Site 2; 2) Waitakere Ranges, Site 3 and; 3) Omakau, Site 6. Indices were standardized by always commencing frog counts 1 -1.5 h after darkness had fallen (as Leiopelma will have left their retreats by this time, given favourable conditions; Cree 1989) and always completing transects within 30 – 40 min. This allowed ample time to spotlight for frogs along the transect, while moving slowly, and provided a count of frogs that could be compared
among nights. At Omakau, transects were walked during the day as southern bell frogs are known to exhibit diurnal basking behavior (Goldingay 2008; Hamer et al. 2010).

5.2.5 Stomach and faecal content analysis

Stomach and faecal samples collected on nights when no frogs were observed were excluded from assays, because whether small mammals are actively hunting frogs, or only consuming them opportunistically, the chance of detecting frogs as prey would be expected to be much greater on nights when frogs had emerged from their refuges. Chapter 3 of this thesis showed that morphological techniques to identify frogs as prey in small mammal diet samples are largely unreliable, particularly in faecal samples. As a consequence of this, faecal samples collected from field sites were not subjected to morphological analysis.

All assayed stomach samples were subject to morphological analysis without the aid of a microscope. A subset (30%) of the samples were examined more thoroughly under a dissecting microscope (Olympus SZ61, with Olympus DP25 digital camera attachment, Olympus Corporation, Tokyo, Japan) at between 6.7 X and 45 X magnification.

DNA-based analysis followed the methods developed in Chapter 4. In short, samples were homogenised and DNA was extracted using either the 'Qiagen DNeasy blood and tissue' kit (Qiagen; stomach contents) or the 'Zymo D6010 Faecal DNA' kit (Zymo Research; faecal contents). PCRs were performed on the extracted DNA, using the species-specific prey primer pairs and protocol developed in Chapter 4. Aerosol-resistant pipette tips were used throughout all PCR procedures. PCR products were separated by gel electrophoresis and DNA bands were subsequently viewed with a blue light transilluminator. All positive PCRs were re-run and amplicons were sequenced using the ABI 3730xl DNA Analyser (Applied Biosystems).
Resultant sequences were aligned with the corresponding 12S gene sequences from all mainland frog species using the BLAST® tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi), the BLASTN 2.2.28 program (Zhang et al. 2000), the MegaBLAST algorithm (Morgulis et al. 2008) and the “Align Sequences Nucleotide BLAST” option (blastn suite). Frog species sequences were obtained either from the Genbank® (Benson et al. 2010) nucleotide database available from the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/) or from sequences obtained in Chapter 4 of this thesis. The species of prey to which a sequence belonged was identified based on the BLAST® score, query cover, associated E-values, and identities of the alignment results.

5.2.6 Statistical analysis

All statistical analyses were carried out using SPSS Version 20.0 (IBM Corporation, IBM SPSS Statistics for Windows, New York, U.S.A.). Following Zippin (1958) and Brown et al. (1996) linear regression was performed to estimate the density of ship rats at the Whareorino Forest and Waitakere Ranges study sites, using the nightly catch versus the cumulative catch. This was not feasible for data from the Omakau study sites. For all sites, trapping success was measured in terms of captures per 100 corrected trap nights (captures$^{-100\text{ctn}}$) and faecal sample collection success in terms of samples per 100 bait tube nights (samples$^{-100\text{btn}}$).

The minimum number of frogs consumed by ship rats at Whareorino Forest and Waitakere Ranges study sites was estimated based on the equation developed by Dempster (Equation 1; 1967; see also Ashby 1974 and; Sopp et al. 1992):

$$ r = pd \times \frac{t_{PA}}{t_{DP}} $$

(1)

where, $r$ = rate of predation (minimum number of prey consumed per unit area), $p =$ proportion of predators containing prey items, $d =$ predator density, $t_{PA} =$ number of days prey available and $t_{DP} =$ detection period.
To include data from multiple sites (using average rat density) and to account for sampling on nights when prey was unavailable (i.e. nights when no frogs were observed), this was modified to (Equation 2):

\[ r = \frac{p \times \sum d_i \times t_{PA}}{n \times t_{DP}} \]  

(2)

where, \( r \) = rate of predation (number of prey consumed per ha per night), \( p \) = proportion of predators containing prey items, \( d_i \) = estimated predator density at each site (per ha), \( n \) = number of sites, \( t_{PA} \) = number of nights prey available, \( t_{PU} \) = number of nights prey unavailable, and \( t_{DP} \) = detection period.

It should be noted that \( t_{PU} \) was taken to be nights on which frogs were not observed (i.e. nights on which frogs tended to remain in their retreats). Frogs may also be at risk from predators while in their retreats, but it was assumed here that this risk would not be as great (see also Section 5.4.8).

Where ship rat density was unavailable (sites where grid trapping had not been carried out), it was assumed to be an average of the other sites within that study area. From Chapter 4, the mean detection period of frogs as prey in ship rat diet samples is estimated as 7.26 h (stomach contents) and 12.89 h (faecal contents) post frog ingestion. Because the number of samples collected varied on different nights and at different sites, \( t_{DP} \) was averaged by Equation 3:

\[ t_{DP} = \frac{(7.26s + 12.89f) + (s + f)}{l} \]  

(3)

where, \( s \) = number of ship rat stomach samples, \( f \) = number of ship rat faecal samples, and \( l \) = average duration of night during study period (h).

Where \( t_{DP} \) was greater than \( l \), the detection period was set to one (i.e. in these cases prey was, on average, detectable for the entire night).
The equation (2) was used a number of times for each study area, by varying: 1) the number of study sites included (e.g. including all sites or only those where frog predation was evident); 2) the number of nights available (i.e. using only diet samples collected on nights frogs were observed, or including samples from all nights) and; 3) the type of samples included (i.e. stomach or faecal or both). Any night that frogs were observed along a transect was taken to be a night that prey were available. The assumptions of this equation (2) are discussed in Section 5.4.7.1.

The frequency with which animals were visiting bait tubes at each sites was calculated as (Equation 4):

\[
\text{visit rate} = \frac{\text{bait tubes with small bait removed}}{\text{number of bait tubes}}
\]  

(4)

5.3 Results

5.3.1 Faecal sample collection

Of the 542 faecal samples collected from bait tubes, the vast majority were ship rat faeces, although some mouse faeces were also present. Ship rats and possums (Trichosurus vulpecula) visiting one bait tube were captured by the motion sensing camera (see Appendix K), and possum faeces were often observed near to, but never inside, bait tubes. Possum faeces were not collected.

The modified bait tube design worked well when targeting rodents with peanut butter bait, achieving an overall visit rate of 79 %, and in the two native forest habitats (Whareorino Forest and Waitakere Ranges) this was 87 %. However, rabbit meat-baited tubes, targeting mustelids, had a visit rate of only 3.83 % (Table 5.1), and none of the faeces collected from these tubes were from mustelids. Peanut butter-baited tubes resulted in the collection of 536 faecal samples over 2,992 bait tube nights (btn; 18 %), of which 106 (20 %) were deposited on nights coinciding with frog emergence (Table 5.1). Rabbit meat-baited tubes resulted in 6
samples (0.8 % of btn; Table 5.1), none of which were deposited on nights coinciding with frog emergence. An additional 10 faecal samples were collected opportunistically from the Omakau sites, which were found near, but not inside, bait tubes. Seven of these were Norway rat faeces and three were from hedgehogs.

5.3.2 Animal captures and rat densities

A total of 230 animals were kill-trapped, including ship rats, Norway rats, mice, stoats Mustela erminea, ferrets Mustela putorius furo and hedgehogs Erinaceus europaeus, of which 156 were captured on nights that frogs had emerged (Table 5.2).

Linear regressions yielded wide confidence intervals and low $R^2$ values for estimated ship rat numbers during the initial trapping sessions, which used 25 m trap spacing (Figure 5.4). Estimates from subsequent sessions, using 20 m trap spacing, had narrower confidence intervals and higher $R^2$ values (Figure 5.4), and are considered to be more reliable estimates. Including all trapping sessions, the estimated density of ship rats across study sites varied from 2.44 to 10.9 rats ha$^{-1}$ (Figure 5.5). However, excluding those initial sessions, rat density did not vary greatly between study sites (5.77 to 6.67 rats ha$^{-1}$; Figure 5.5).

The variability of nightly frog emergence during collection periods (Range = 0 – 0.45 frogs m$^{-1}$; Figures 5.6 - 5.8) resulted in many stomach and faecal samples being collected on nights when no frogs were observed and, while these samples were not subject to diet analysis, predation rate was estimated both excluding and including these samples, with the assumption that all were negative for frogs as prey (detailed in Section 5.3.4).
Table 5.1  Number of bait tubes at each study site, number of nights bait tubes were in place and number of faecal samples collected. Bait tube nights (btn) = no. of bait tubes x no. of nights. P – baited with peanut butter, R – baited with rabbit meat. All samples were collected between November 2011 and March 2012 (faecal samples were not collected during the 2010 field sessions). An additional 10 faecal samples (7 of Norway rat and 3 of hedgehog) were collected opportunistically (found away from bait tubes) from Omakau Site 6 – these are not included in this table.

| Study area          | Site | Nights | No. of bait tubes (P|R) | btn (P|R) | Bait taken | Faecal samples collected (P|R) | Samples \(-100 \text{ btn}(P|R)\) | Samples on nights frogs were observed |
|---------------------|------|--------|------------------------|-----------|------------|-------------------------------|--------------------------------|-----------------------------------|
| Whareorino Forest   | 1    | 11     | 41 | 5                       | 451 | 55         | 259 | 0                     | 126 | 0                                      | 27.94 | 0                                   | 40 | 0                                  |
| Whareorino Forest   | 2    | 7      | 41 | 5                       | 287 | 35         | 170 | 0                     | 84  | 0                                      | 29.29 | 0                                   | 11 | 0                                  |
| Waitakere Ranges    | 3    | 18     | 32 | 7                       | 576 | 126        | 572 | 0                     | 101 | 4                                      | 17.53 | 3.17                                | 14 | 0                                  |
| Waitakere Ranges    | 4    | 22     | 32 | 7                       | 704 | 126        | 663 | 7                     | 49  | 0                                      | 6.96  | 0                                    | 8  | 0                                  |
| Waitakere Ranges    | 5    | 22     | 32 | 7                       | 704 | 126        | 701 | 2                     | 176 | 2                                      | 25.0  | 1.59                                 | 58 | 0                                  |
| Omakau              | 6    | 13     | 10 | 10                      | 130 | 130        | 1   | 3                     | 0   | 0                                      | 0    | 0                                    | 0  | 0                                  |
| Omakau              | 7    | 14     | 10 | 10                      | 140 | 140        | 2   | 8                     | 0   | 0                                      | 0    | 0                                    | 0  | 0                                  |
Table 5.2 Number and species of animals kill-trapped at respective study sites. Bracketed numbers indicate captures that occurred on nights when frogs were observed along the frog abundance transects. Capture rates are expressed per 100 corrected snap trap nights (ctn), unless otherwise indicated. Correction excludes traps that were sprung, had bait removed, or were missing. Species trapped included ship rat (SR), Norway rat (NR), mouse (M), stoat (ST), ferret (F) and hedgehog (H).

<table>
<thead>
<tr>
<th>Study area</th>
<th>Site</th>
<th>Session</th>
<th>Trap nights (Snap traps</th>
<th>DOC traps)</th>
<th>Snap trap captures (captures on nights frogs were observed)</th>
<th>DOC trap captures (captures on nights frogs were observed)</th>
<th>Captures$^{-100}$ ctn</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whareorino Forest</td>
<td>1</td>
<td>Mar 2010</td>
<td>405</td>
<td>25</td>
<td>SR - 16 (16)</td>
<td>None</td>
<td>SR - 5.95</td>
</tr>
<tr>
<td>Whareorino Forest</td>
<td>2</td>
<td>Mar 2012</td>
<td>405</td>
<td>25</td>
<td>SR - 44 (1); M - 1 (0)</td>
<td>None</td>
<td>SR - 12.36; M - 0.28</td>
</tr>
<tr>
<td>Waitakere Ranges</td>
<td>3</td>
<td>Apr 2010</td>
<td>405</td>
<td>25</td>
<td>SR - 41 (30)</td>
<td>None</td>
<td>SR - 12.54</td>
</tr>
<tr>
<td>Waitakere Ranges</td>
<td>3</td>
<td>Dec 2011</td>
<td>405</td>
<td>25</td>
<td>SR - 51 (34); M - 2 (0)</td>
<td>None</td>
<td>SR - 13.46; M - 0.79</td>
</tr>
<tr>
<td>Waitakere Ranges</td>
<td>4</td>
<td>Dec 2011</td>
<td>405</td>
<td>25</td>
<td>SR - 42 (42); M: - 2 (2); ST - 8 (8)</td>
<td>None</td>
<td>SR - 16.87; M - 0.8; ST - 3.21</td>
</tr>
<tr>
<td>Waitakere Ranges</td>
<td>5</td>
<td>Dec 2011</td>
<td>15</td>
<td>0</td>
<td>SR -11 (11)</td>
<td>None</td>
<td>SR - 78.57$^1$</td>
</tr>
<tr>
<td>Omakau</td>
<td>6</td>
<td>Feb 2010</td>
<td>300</td>
<td>85</td>
<td>NR - 3 (3); M - 5 (5)</td>
<td>ST - 1 (1); H - 2 (2);</td>
<td>NR – 1.71; M – 2.84; F - 1 (1)</td>
</tr>
<tr>
<td>Omakau</td>
<td>7</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td></td>
</tr>
</tbody>
</table>

$^1$ This result is derived from one night of opportunistic trapping using 15 snap rat traps only and is not comparable with other results presented here.

$^2$ Figure based on DOC traps.
Figure 5.4 Standard linear regressions for nightly catch of ship rats against cumulative number of captures, to estimate total number of rats in trapping area. Bold line indicates predicted model values, dashed lines show 95% confidence intervals. Asterisks indicate initial trapping session with 25 m trap spacing.
Figure 5.5 Estimated density of rats (rats ha\(^{-1}\)) at the four sites investigated (on 5 trapping sessions). Error bars show 95% confidence intervals (CI). Values above each bar indicate the density estimate from linear regressions (lower 95% CI, higher 95% CI; R\(^2\) value). Note that 2010 trapping sessions used 25 m trap spacing while subsequent sessions used 20 m trap spacing.
Figure 5.6 Whareorino Forest study area – 2010 and 2012 Sessions. Frog emergence versus diet sample collection periods. ♦ represents the rate of frog encounters (frogs m⁻¹) each night along the monitored transect (transects were not searched during extended dry periods). Thick horizontal lines indicate the period during which trapping (solid lines) or faecal sample collection (dashed lines) was undertaken at each site. Day 1 in 2010 represents the 21<sup>st</sup> March; Day 1 in 2012 represents 18<sup>th</sup> February.
Figure 5.7 Waitakere Ranges study area – 2010 and 2011 Sessions. Frog emergence versus diet sample collection periods. ♦ represents the rate of frog encounters (frogs m⁻¹) each night along the monitored transect (transects were not searched during extended dry periods). Thick horizontal lines indicate the period during which trapping (solid lines) or faecal sample collection (dashed lines) was undertaken at each site. Day 1 in 2010 represents the 4th April; Day 1 in 2011 represents 9th November. Note that trapping at Site 5 was only carried out on one night (■).
Figure 5.8 Omakau study area – 2010 and 2012 Sessions. Frog emergence versus diet sample collection periods. ♦ represents the rate of frog encounters (frogs m⁻¹) each night along the monitored transect. Thick horizontal lines indicate the period during which trapping (solid lines) or faecal sample collection (dashed lines) was undertaken at each site. Day 1 in 2010 represents the 23rd February; Day 1 in 2012 represents the 11th January.
5.3.3 Detecting frogs in the diet of small mammals in New Zealand

A total of 5 diet samples tested positive (1.8 %, n = 272 assayed stomach and faecal samples) for the presence of frogs as prey (Table 5.3). Four of these were from stomach samples (2.6 %, n = 156 assayed stomach samples). Using morphological diet analysis, none of the ship rat stomach contents were found to contain remains of frogs. It was only using DNA-based analysis that these predation events were detected. At Omakau, one hedgehog stomach sample and one hedgehog faecal sample (collected opportunistically during study period) tested positive for southern bell frog. BLAST results showing the similarity of unknown prey sequences to all frog species present on the New Zealand mainland are provided in Appendix L (over 97 % identical in all cases).

Table 5.3 Samples positive for the presence of frogs as prey and the method by which prey could be detected. Faeces were subject only to DNA-based analysis, due to the very low probability of detecting frog remains using morphological analysis detailed in Chapter 3.

<table>
<thead>
<tr>
<th>Study Area</th>
<th>Site</th>
<th>Session</th>
<th>Prey species</th>
<th>Predator</th>
<th>Sample type</th>
<th>Detection method success</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whareorino Forest</td>
<td>1</td>
<td>2010</td>
<td><em>L. archeyi</em></td>
<td>ship rat</td>
<td>stomach</td>
<td>No</td>
</tr>
<tr>
<td>Whareorino Forest</td>
<td>2</td>
<td>2012</td>
<td><em>L. archeyi</em></td>
<td>ship rat</td>
<td>stomach</td>
<td>No</td>
</tr>
<tr>
<td>Waitakere Ranges</td>
<td>3</td>
<td>2011</td>
<td><em>L. hochstetteri</em></td>
<td>ship rat</td>
<td>stomach</td>
<td>No</td>
</tr>
<tr>
<td>Omakau</td>
<td>6</td>
<td>2010</td>
<td><em>L. raniformis</em></td>
<td>hedgehog</td>
<td>stomach</td>
<td>Yes</td>
</tr>
<tr>
<td>Omakau</td>
<td>6</td>
<td>2010</td>
<td><em>L. raniformis</em></td>
<td>hedgehog</td>
<td>faeces</td>
<td>NA</td>
</tr>
</tbody>
</table>

5.3.4 Estimating predation rate of ship rats on native frogs

At Whareorino Forest a total of 270 ship rat diet samples were collected across 2 sites on 24 nights (5 in 2010 and 19 in 2012). Of these, 68 were collected on nights that frogs were observed (referred to as \( t_{PA} \); nights prey available; n = 10) and only these samples were
assayed for the presence of frogs as prey. Two stomach samples (one from Site 1, 2010 and one from Site 2, 2012) were positive for Archey’s frog (2.94 % of all assayed samples; 11.76 % of assayed stomach samples). If only considering $t_{PA}$, the range of predation rate estimates is $0.09 – 0.9$ frogs/ha/$t_{PA}$ (Table 5.4). When considering all samples collected, and assuming the predation rate to equal zero on nights when frogs were not observed ($t_{PU}$), this range drops to $0.01 – 0.25$ frogs/ha/night ($t_{PA} + t_{PU}$; Table 5.4). Using the data from assayed samples across all sites and sessions, the estimated number of frogs eaten over the effective study area (17.93 ha) and study duration (10 $t_{PA}$) was between 25.1 and 161.4. Extending this to non-assayed samples, again assuming predation rate to equal zero on $t_{PU}$, leads to estimates of 13.75 – 27.5 frogs consumed (24 nights).

At the Waitakere Ranges a total of 471 ship rat diet samples were collected across 3 sites on 38 nights (5 in 2010, 33 in 2012). Of these, 197 were collected on nights that frogs were observed ($t_{PA} n = 15$) and only these samples were assayed for the presence of frogs as prey. One stomach sample (from Site 3, 2011) was positive for Hochstetter’s frog (0.51 % of all assayed samples; 0.86 % of assayed stomach samples). If only considering $t_{PA}$, the range of predation rate estimates is $0.04 – 0.26$ frogs/ha/$t_{PA}$ (Table 5.4). When considering all samples collected this range drops to $0.01 – 0.05$ frogs/ha/night ($t_{PA} + t_{PU}$; Table 5.4). Using the data from assayed samples across all sites and sessions, the estimated number of frogs eaten over the effective study area (22.92 ha) and study duration (15 $t_{PA}$) was between 13.8 and 89.4. Extending this to non-assayed samples leads to estimates of 8.7 – 34.8 frogs consumed (38 nights).
Table 5.4 Minimum estimated predation rates (frogs per ha per night) by ship rats at Whareorino Forest (Archeys frog) and the Waitakere Ranges (Hochstetter’s frog) study areas. Varying estimates are shown by site and by sample type included. Diet samples were collected on nights when frogs were observed (tPA) and on nights frogs were not observed (tPU), n = number of samples for each category. Samples from tPU were not assayed for the presence of frogs as prey and are all assumed to be negative. Note that no faeces were positive for frogs as prey. Also, no samples (stomach or faecal) from sites 4 or 5 were positive for frogs as prey.

<table>
<thead>
<tr>
<th>Study area</th>
<th>Site</th>
<th>Session(s)</th>
<th>Stomachs collected on tPA</th>
<th>Stomachs collected on tPA+tPU</th>
<th>Stomachs and faeces collected on tPA</th>
<th>Stomachs and faeces collected on tPA+tPU</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whareorino Forest</td>
<td>1</td>
<td>2010</td>
<td>0.25 (16)</td>
<td>0.25 (16)</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>2012</td>
<td>9.46 (1)</td>
<td>0.04 (44)</td>
<td>0.29 (20)</td>
<td>0.01 (128)</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>2010+2012</td>
<td>0.25 (16)</td>
<td>0.25 (16)</td>
<td>0.09 (48)</td>
<td>0.03 (142)</td>
</tr>
<tr>
<td></td>
<td>1, 2</td>
<td>2010+2012</td>
<td>0.90 (17)</td>
<td>0.15 (60)</td>
<td>0.14 (68)</td>
<td>0.01 (270)</td>
</tr>
<tr>
<td>Waitakere Ranges</td>
<td>3</td>
<td>2010</td>
<td>0 (30)</td>
<td>0 (41)</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>2011</td>
<td>0.26 (34)</td>
<td>0.03 (51)</td>
<td>0.15 (48)</td>
<td>0.01 (152)</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>2010+2011</td>
<td>0.18 (64)</td>
<td>0.05 (92)</td>
<td>0.13 (78)</td>
<td>0.01 (193)</td>
</tr>
<tr>
<td></td>
<td>3, 4, 5</td>
<td>2011</td>
<td>0.10 (87)</td>
<td>0.05 (104)</td>
<td>0.04 (167)</td>
<td>0.01 (430)</td>
</tr>
<tr>
<td></td>
<td>3, 4, 5</td>
<td>2010+2011</td>
<td>0.08 (128)</td>
<td>0.04 (145)</td>
<td>0.04 (208)</td>
<td>0.01 (471)</td>
</tr>
</tbody>
</table>

\(^1\)This estimate is included here for the sake of completion, but is based on only one assayed sample. This was excluded from predation estimate ranges mentioned elsewhere in the text.
Chapter 5 – Predation on frogs in New Zealand

5.4 Discussion

This is the first time that New Zealand native frogs have been detected as prey in mammalian stomach contents. The DNA-based methods for detecting prey that were developed under laboratory conditions (Chapter 3) were validated for field-collected samples and were successful where morphological analysis was not. This is the first time that the impact of introduced predators on New Zealand frogs has been estimated based on diet analysis. Ship rats are consuming both of the two species of *Leiopelma* still present on mainland New Zealand.

5.4.1 Predation estimates on New Zealand native frogs

The range of minimum predation rate estimates during the study period is wide (Archey’s frog: 0.01 – 0.9 frogs/night/ha; Hochsetter’s frog: 0.01 – 0.26 frogs/night/ha). Two monitored grids (each 100 m$^2$) c. 200 m from Sites 1 and 2 in Whareorino Forest provided estimates of 33.9 and 76.8 frogs per 100 m$^2$ in 2011 (Pledger 2011). If these grids are considered representative of the greater area across the trapping grids in the present study, then the minimum proportion of this Archey’s frog population depredated by ship rats during the study period (totalling 24 days) was between 0.003 % and 0.27 %. Unfortunately, without having more data, this large range is difficult to interpret and these rates are very likely to change over time, given the annual fluctuation of rat densities (e.g. Daniel 1972 and; Smith 1986), the varying food sources available to rats, and the seasonal variation in nights suitable for frog emergence (frogs will still emerge from retreats during suitable winter nights; Cree 1989). However, this is the first time such data has been collected and it gives us a snapshot of potential predation rates during the study period.

Estimating the predation rate on Hochstetter’s frog using a per ha scale may not be the most accurate method as these frogs are usually confined to the linear habitats of small streams and seepages (McLennan 1985; Newman and Towns 1985; Green and Tessier 1990; Tessier *et al.*
Therefore, an estimate of the proportion of Hochstetter’s frogs depredated cannot be considered reliable. Attempting to do so, and taking the effective trapping area as a 312 m reach of each stream (the diameter of the effective trapping grid centred on the stream), the predation rate becomes 0.002 – 0.083 frogs/night/100 m. The relative abundances of frogs at these sites in 2008 were: Site 3 - 42.5 frogs\textsuperscript{100m} – Site 4 – 5.6 frogs\textsuperscript{100m} – Site 5 - 40 frogs\textsuperscript{100m} (Moreno 2009). This equates to an estimate of the minimum proportion of frogs depredated by ship rats of between 0.92 % and 22.23 % of the population during the study period (38 days). Again, without more reliable data (both for predation rates and relative abundances) these estimates must be treated with caution.

### 5.4.2 Comparison to other studies

This is the first time that either Archey’s frog or Hochstetter’s frog has been detected in the stomach contents of any mammal. It is notable that where traditional methods of visually identifying prey remains failed, DNA-based methods succeeded. This might be because ship rats often do not ingest skeletal components of frog prey, sometimes preferring to consume only soft tissue (Chapter 2; and see Egeter et al. 2011).

Using stable isotope analysis Najera-Hillman et al. (2009) concluded that shortfin eels \textit{(Anguilla australis)} and banded kokopu \textit{(Galaxias fasciatus)} may be predators of Hochstetter’s frogs in the Waitakere Ranges, while the data for ship rats were inconclusive. Also in the Waitakere Ranges, Nájera-Hillman et al. (2009b) found no difference in the relative abundance or size class of Hochstetter’s frogs between streams that had no rodent control and those that where rodent numbers were very low (following 7 years of pest management). Conversely, Mussett (2005) and (Baber et al. 2008) found that Hochstetter’s frog abundance was higher in pest controlled areas than in non-pest controlled areas in the Hunua Ranges. The results of Mussett (2005) were complicated by the fact that in some pest
control sites, rat abundance was similar to that in non-pest control sites. Also, among pest control sites the highest rat abundance coincided with the highest frog abundance.

A major difficulty with comparing frog abundance estimates (e.g. at sites with and without mammal control) is that a difference in abundances may not reflect a difference in population size, but only in detection probability (Buckland et al. 2000; Yoccoz et al. 2001; Crossland et al. 2005), which can vary spatially or temporally (Anderson 2001; Hyde and Simons 2001; Anderson 2003; Bailey et al. 2004; Crossland et al. 2005). For example, Hochstetter’s frogs are found both in portions of streams with a high percentage cover of ground riparian vegetation and portions with very little ground vegetation (pers. obs.). Finding frogs is much more difficult where vegetation cover is higher, so attempting to directly compare the frog abundances between the two streams would not be effective without an estimate of detection probability. Predation estimates have the advantage over frog counts of not being affected by frog detection probability or observer bias. McLennan (1985) calculated a four-fold difference in abundances of Hochstetter’s frogs based on results collected from different observers.

Between 2005 and 2011 the Department of Conservation ran a predator removal experiment at Whareorino Forest whereby four 10 m x 10 m Archey’s frog population monitoring grids were set up, two in a treatment area poisoned for rodents and two in a non-treatment area (Pledger 2011). Over that period frog population sizes remained stable or increased on grids inside the treatment area and decreased in the non-treatment area (Pledger 2011). This supports the theory that rodents have a detrimental effect on Archey’s frogs. The experiment was limited to only two relatively small grids within each (much larger) treatment block and it is possible that other factors may have influenced the results. One potential influencing factor could be site differences in the abundances of other known frog predators such as stoats, ferrets, pigs (Sus scrofa) or green and golden bell frogs (Litoria aurea; see Appendix A for a full list of known predators of frogs in New Zealand). The DOC monitoring data was also
incorporated into another mark-recapture model (Ohmer et al. 2012) that included predation (as per treatment block) and disease (frogs testing positive for chytridiomycosis) as factors. The authors found no functional effect of predator control and found that the population appears to stable (Ohmer et al. 2012).

An ideal situation to determine predator impact through frog indices or density might involve a before and after approach (as noted by Pledger 2011), where frog monitoring commences before predator removal and monitoring continues for some time after. Vredenburg (2004) successfully used this approach to determine the recovery of frogs (Rana muscosa) following removal of rainbow trout (Oncorhynchus mykiss) and brook trout (Salvelinus fontinalis). The primary problem for New Zealand’s native frogs is that they are long-lived (Leiopelma pakeka reaching >37 years; Bell and Pledger, 2010) produce few eggs (1 - 22 eggs; Bell and Wassersug 2003) relative to many other amphibians, and do not reproduce every year, so the before and after approach would necessitate a very long-term monitoring plan to detect population trends.

5.4.3 Can native frogs sustain this level of predation?

The present study shows that ship rats do prey on native frogs. However, the two species remaining on the mainland persist in the presence of relatively high densities of ship rats. It is difficult to say whether ship rat predation alone threatens native frogs with extinction, given the wide estimates. Roughly estimated, the data on Archey’s frogs from Pledger (2011) suggest an annual recruitment of anywhere between 881 and 3148 frogs per hectare in the presence of rodents, while extrapolating the data presented herein to an annual rate, suggests anywhere between 1.7 and 315 frogs depredated by ship rats per hectare (0.05 % and 4.11 % of the overall population). This indicates that ship rat predation alone would not be enough to render the population extinct. Recruitment estimates are not available for Hochstetter’s frogs, but predation rates may be higher (extrapolating to an annual rate of 9.5 to 30.3 frogs per 100
m stream reach). Of course, these are very rough estimations and do not include the range of other threats faced by native frogs, such as disease, nor their natural death rate (see also Section 5.4.7 – Considerations and caveats) and as such must be treated with caution. As more data become available on the life histories and population dynamics of native frogs, as well as on the predation rates by predators, it is expected that these could be incorporated into population viability analyses and mark-recapture studies to gain a reliable and accurate picture of the impacts that introduced predators are having.

### 5.4.4 Other notable field observations

Green and golden bell frogs are known to prey on Archey’s frog (Thurley and Bell 1994b) and this species was noted on two occasions within c. 10 m of known Hochstetter’s frog locations (pers. obs.). It is possible that these frogs may also represent a threat to Hochstetter’s frogs. Evidence of pig presence (faeces, footprints and trampling) was common at all native forest sites. Pigs are known to consume green and golden bell frogs in New Zealand (Egether, B. and Krull, C, unpublished) and are likely to prey on native frogs.

### 5.4.5 Rat (and trap) density

Rat densities at the study sites were comparable to those found in other studies in New Zealand forests (Daniel 1972; Innes et al. 1992; Dowding and Murphy 1994; Hooker and Innes 1995; Brown et al. 1996; Blackwell et al. 2002; Wilson et al. 2007; Innes et al. 2010). Trapping was carried out in autumn at Whareorino Forest, as was the first trapping session in the Waitakere Ranges, and this may account for some of the higher densities estimated, as Daniel (1972) and Smith (1986) found that rat density was highest at this time of year.

The effective trapping area was calculated by adding a 56 m boundary to the actual trapping grid area (after Dice 1938), based on the mean ship rat home range at Rotoehu Forest (Central North Island, New Zealand; Hooker and Innes 1995). It was outside the scope of this study to measure ship rat home ranges, and this boundary-adding method has been used in other
studies (Brown et al. 1996; Blackwell et al. 2002). However, ship rat home range sizes are known to vary between sexes (Innes et al. 1992; Hooker and Innes 1995), as well as over time (e.g. Daniel 1972 and; Smith 1986) and between habitats, and using a constant boundary strip is not the most reliable method (Efford 2004).

Reducing the trap spacing from 25 m to 20 m greatly improved the precision of the rat density estimates in this study. This is most likely due to a relationship between rat density and trap density (aspects of which are discussed by Wilson et al. 2007). The grid layout was a scaled-down version of that implemented by Brown et al. (1996; and followed by Blackwell et al. 2002) who used 169 traps in a 17 ha effective trapping area. Their grids therefore had 9.9 traps ha⁻¹, whereas the initial grids in the present study had only 7.43 traps ha⁻¹. Once the spacing had been reduced the density of traps increased to 10.6 ha⁻¹ and linear model fits improved.

Where similar trapping grids are being used I do not recommend using a trap density lower than that used by Brown et al. (1996) and Blackwell et al. (2002).

5.4.6 Faecal collection

The modified bait tube design proved to be a very effective method for collecting rat faeces in forest habitats, however, no faeces were deposited in tubes, either in pastoral habitat, or when targeting mustelids in forest habitat. This is highlighted by the fact that seven fresh Norway rat faeces were found during the study period at Omakau (Site 6; pastoral habitat), near to but not inside, bait tubes. Also, eight stoats were trapped at Site 4 in the Waitakere Ranges, but no mustelid faeces were deposited at bait tubes during the 22 days prior to trapping there.

Prior to this study bait tubes have largely been used simply to detect the presence of small mammals (Churchfield et al. 2000; Greenwood et al. 2002; Carter and Churchfield 2005; Poulton and Turner 2009; Scott et al. 2012). This does not necessarily require individuals to make repeat visits or deposit multiple faecal pellets. The modified design seems to be
effective at promoting both of these behaviours, while also limiting the amount of bait consumption (and therefore potentially yielding more dietary information).

It is also interesting to note that in the Waitakere Ranges, Sites 3 and 4 had very similar rat densities, the same number of bait tube nights and they achieved similar bait tube visit rates (99 % and 94 % respectively). However, the number of faecal samples collected at Site 4 was only half that at Site 3. Stoats were present at Site 4 (Table 5.2) and that site had the highest number of baits taken from tubes baited with rabbit meat (presumably taken by the stoats). It is possible that the presence of stoats influenced the nightly activity of rats in this area, by reducing the amount of time rats stayed at bait tubes, accounting for the large difference in number of faecal samples collected. If this is the case, the presence of stoats may be decreasing the amount of time that ship rats spend at ground level (ship rats spend c. 75 % of their time in trees, 2 m above ground level; Hooker and Innes 1995), or may be decreasing the amount of time spent in any one place while at ground level. In any case, this is an unexpected result and could be an important consideration for other rat trapping or monitoring studies.

5.4.7 Considerations and caveats

The presence of animal material in the stomach or faecal contents of a predator may be the result of scavenging rather than direct predation (Symondson 2002; Harper et al. 2005) and has been shown to be a cause of false positives in invertebrate diet studies (Foltan et al. 2005; Juen and Traugott 2005). Ship rats are known to scavenge (Innes et al. 1996; Innes 2001; Stapp 2002) and this could have affected results in the present study. The rate of scavenging will primarily depend on the availability of carcasses (Juen and Traugott 2005; King et al. 2008), which in this case is unknown. Fitzgerald and Campbell (2002) noted that the bite mark injuries on Archey’s frogs found at Whareorino Forest appeared to be the cause of death, rather than being inflicted post-mortem.
Another source of error could be the detection of secondary predation (Sheppard and Harwood 2005; King et al. 2008), where one predator consumes another predator, shortly after the latter had consumed the target prey. Secondary predation possibilities for the present study could include a mouse or invertebrate that had partially ingested a frog, prior to then itself being ingested by a ship rat. This problem can be exacerbated where prey can be detected for long periods. In one example, prey of a spider (*Tenuiphantes tenuis*) was detectable for c. 60 h, and the same prey was detectable in beetles that ingested the spiders (Sheppard and Harwood 2005). This suggests that the probability of detecting secondary predation in the present study is not high, given the relatively short detection periods of < 1 night.

One feature obscured by using DNA-based methods is the life stage of the prey. It may have been the case that eggs, tadpoles (although Archey’s frog lacks a free-swimming tadpole stage; Stephenson 1951b; 1961) or young juveniles were the prey from which DNA was amplified in the present study. Hochstetter’s frog eggs (clutches of 10 – 22; Bell and Wassersug 2003) are laid from September to May, with young frogs emerging from December – August (Beauchamp et al. 2010), while eggs of Archey’s frogs have been found between November and January (Stephenson and Stephenson 1957; Thurley and Bell 1994a). Because this study is the first to detect native frogs in mammal stomach contents, there is no data available to suggest rats target particular life stages, but it should be considered when investigating DNA-based diet analysis. If native frogs were not of such a high conservation value, trials could be carried out using *Leiopelma* as prey to assess whether eggs, young or adults are preferred.

Finally, contamination (environmental or during PCR) in DNA-based studies can be controlled for, but can never be fully eliminated. Environmental contamination may have been possible for faecal sample collection. The only positive for frogs as prey in faeces was a
hedgehog faecal sample collected near a southern bell frog breeding pond. Ficetola et al. (2008) and Thomsen et al. (2012) successfully used PCR techniques to detect the presence of amphibians in a number of freshwater habitats using only water samples. It is possible therefore that pond water may have contaminated this sample leading to a false positive. Environmental contamination of stomach samples seems much more unlikely, as full predator carcasses were removed from the sites before being subjected to careful dissection using instruments washed in ethanol and flamed. Also, whole stomachs were taken, reducing the risk of contamination in the field and stomach contents were only emptied under laboratory conditions. Contamination during PCR is also possible, however, every eight PCRs included a negative control (only reagents). The lack of amplification in these sample wells showed that a systematic contamination was not occurring, and contamination of samples without nearby samples also being contaminated would be difficult to explain. As part of a collaborative project, separate from the research presented in this thesis, investigating the use of group-specific primers targeting all frog species as prey, small mammal stomach and faecal samples were obtained from Australia (see Appendix J). The primers amplified prey DNA from species that had never previously been imported into the laboratory used, so contamination was not possible. This supports the idea that positive DNA amplifications in the present study were indeed from prey ingested by predators, rather than an artefact of contamination.

5.4.7.1 Assumptions of predation estimate equation
The predation rates reported in Section 5.3.4 deserve some attention as it is difficult to accurately extrapolate such rates from so few positive results. There are a number of assumptions to be made when using the formula provided by Dempster (1960; 1967).

Firstly, each positive must represent only one prey item. While this likely holds true for this study, as the prey detection period is less than one night, it may be the case that a ship rat will
consume multiple frogs per night (or parts of multiple frogs, as would be indicated from results detailed in Chapter 2). In any case, estimates provided here can only be regarded as minimum number of prey eaten. Secondly, prey detection rates should be similar to those observed in a laboratory. The prey DNA detection period exceeded known gastrointestinal transit times in all laboratory feeding trials (Chapter 4), so it is unlikely that prey transit would be longer in wild mammals. Transit times may be shorter in the wild, but using the laboratory-derived half-live estimates in the predation rate equations was considered a conservative approach, more likely to underestimate than overestimate actual predation rates.

Primers targeting different length fragments can lead to very different DNA detection half-lives (Symondson 2002; Deagle et al. 2006; Pompanon et al. 2012), but all primers used targeted similar sized fragments to those used under laboratory conditions (Chapter 4). Nightly temperatures in the field (usually 10 – 19 °C) did not often exceed that used during laboratory feeding trials (20 °C) and should not have caused a considerably greater DNA degradation in stomach or faecal samples. Trapped rats (and subsequently stomach contents) were treated very similarly under laboratory and field conditions (i.e. c. 8 h left to sit at c. 20 °C, followed by dissection), and environmental factors are unlikely to have had a significant effect on prey detectability. However, none of the ship rat faecal samples yielded positive results for frogs as prey. This may have been an accurate result, or it may be that rats had indeed consumed frogs, but environmental conditions (particularly the wet weather that coincided with frog emergence) played a role in further degrading the prey DNA present in faeces, decreasing the ability to detect predation events. This warrants further study (see Section 5.4.8 for recommendations). Thirdly, the model does not take into account the effects of different numbers of prey available each night. This would be exceedingly difficult to estimate and would need to incorporate a mark-recapture study synchronously with collection of predator stomach and/or faecal samples. The proportion of Archey’s frogs remaining in their retreats for the duration of four night long mark-recapture sessions ranged from 0 to 34
% (Pledger 2011). On the nights that frog predation was recorded in the present study, frog emergence was close to average. So, at least, it can be stated that ship rats are not depredating frogs only on nights with exceptional frog emergence, but are likely to be depredating them on more “usual” frog nights also.

5.4.8 How can predation estimates be refined?

The primary reasons for the wide predation estimates are: 1) It is not known whether rats will prey on frogs while frogs are in their retreats (i.e. on nights when frog emergence is not observed); 2) Detection of frogs as prey in small mammal faeces may be much more difficult in the field than under laboratory conditions and; 3) The low number of samples containing frogs as prey led to some sites with a zero predation rate.

While it is considered unlikely that ship rats are depredating native frogs while frogs are in their retreats, it could be occurring. One way to survey for this would be to analyse all the stomach and faecal samples collected on nights when frogs were not observed. This was beyond the resources of this PhD, but samples are in storage and could be analysed in the future.

In general, the frequency of negative results in DNA detection is higher in field studies than in laboratory studies (Deagle et al. 2005b). The fact that none of the ship rat faecal samples tested positive for frogs as prey, particularly when compared to the 12 % positive for assayed stomach samples at Whareorino, may be due to environmental degradation of the samples. This warrants further study. Oehm et al. (2011) found that bird faeces placed within plastic tubes produced a higher rate of prey DNA detection than those placed on soil, leaves or branches, but all were negatively affected by exposure to sun and rain. Conducting prey feeding trials similar to those outlined in Chapters 3 and 4 of this thesis with ship rats as predators and allowing a proportion of subsequent faecal samples to be subjected to environmental conditions representing those in the wild, would allow researchers to gauge
these effects. It may be that faecal samples can be reasonably excluded from analysis completely, or it may be that the effects could be accounted for by a revision of the prey detection period ($t_{DP}$). Southern bell frog DNA was detected in hedgehog faeces collected after a rainy night, but the degree to which faeces are affected is unclear.

It is considered unlikely that predation on frogs by ship rats was truly zero at some sites, given that they prey on frogs at nearby sites. Including these sites in analyses reduces the predation estimates greatly, but they cannot be reasonably excluded. The collection of more stomach and faecal samples and subsequent diet analysis could help elucidate the actual situation at these sites, as well as providing more robust data for predation estimates overall.

5.4.9 Conclusions

The DNA-based methods, developed under laboratory conditions, for detecting frogs as prey in small mammal stomach and faecal samples, were validated for field-collected samples and were successful where morphological analysis was not. Predation by introduced mammals on native frogs has been observed, where it would otherwise have been overlooked. Ship rats are consuming both species of *Leiopelma* still present on mainland New Zealand, and for the first time, an attempt at quantifying their impact through diet analysis has been made. As more data become available on the life histories and population dynamics of native frogs, as well as on the predation rates by predators, it is expected that reliable and accurate population viability analyses will be developed to assess the impacts of introduced predators.
Chapter 6  General discussion

We are currently in a period of major global biodiversity loss and the decline of amphibians represents the most dramatic example of vertebrate extinction taking place (see Bishop et al. 2012). The impacts of introduced species are one of the primary causes of this loss (Kats and Ferrer 2003; Chanson et al. 2008) and these are often measured using predation rate estimates based on diet studies (Park 2004). The overall goal of this thesis was to aid global amphibian conservation by assessing, developing and utilising tools to determine the presence and extent of frog predation by introduced mammals. To do this, I examined the feeding behaviour of small mammals when ingesting frogs and the potential for traditional morphological analysis and DNA-based diet analysis to detect frogs as prey in small mammal stomach and faecal contents. Using this information I have estimated the predation rate of an introduced small mammal species (ship rat *Rattus rattus*) on two species of native New Zealand frog, Archey’s frog *Leiopelma archeyi* and Hochstetter’s frog *Leiopelma hochstetteri*, which are ranked as the 1st and 39th most Evolutionarily Distinct and Globally Endangered (EDGE 2008) amphibian species in the world (Chapter 5). The detection of these prey species would have gone unobserved using only morphological diet analysis, and a subsequent estimation of predation rate would have been impossible.

This is the first time that: 1) the detection rate of amphibians as prey in mammal stomach and faecal contents has been measured; 2) the detectability of prey DNA has been measured over time in mammal stomach contents; 3) the detectability of prey DNA has been compared, between stomach and faecal contents, over time in any vertebrate; 4) predation rates on native amphibians have been measured using DNA-based diet analysis; 5) New Zealand native frogs have been identified as prey in introduced mammal stomach contents.
The results of these novel investigations have led to some key findings. Traditional techniques used to detect predation, particularly evidence from frog carcasses and morphological analysis of predator diets, have major shortcomings and biases when attempting to determine either the mammal species responsible or the frog species ingested (Chapters 2 and 3). DNA-based diet analysis offers major advantages over traditional methods, with fewer biases, and the detection of prey DNA was limited primarily by its presence, rather than the degradative effects of small mammal ingestion or digestion (Chapter 4). Introduced mammals are preying on native frogs in New Zealand (Chapter 5) and, given other key findings, mammals are likely to have previously been underestimated as predators of amphibians worldwide. These findings, along with the fact that the research presents a number of pioneering investigations, have major implications for both diet analysis studies and global amphibian conservation.

**6.1 Is DNA-based diet analysis the method of choice to determine the impact of introduced predators on amphibians?**

Major advances have recently been made in the field of DNA-based diet analysis, particularly in detecting prey species where traditional methods are unreliable (Deagle et al. 2005b; Boyer et al. 2013). For the first time, I have demonstrated that these techniques can be developed and applied to amphibians as prey of introduced mammals, and that they offer major advantages over traditional methods (Chapter 4). Other studies have also shown an increase in prey detection success using DNA-based methods over morphological methods (see Casper et al. 2007b; Soininen et al. 2009; Dunn et al. 2010), although the disparity in success between methods is particularly pronounced in the present study, and this is likely due to the fact that morphologically diagnostic frog material may not even be ingested by small mammals (Chapter 2) or, if it is ingested, it does not survive ingestion particularly well (Chapter 3).
Direct observation of predation events is not always possible, especially when observing small or elusive animals (such as amphibians) with cryptic food-web ecology (Sheppard and Harwood 2005). There are a suite of other tools that can be used to detect predation and infer the impact of predators on prey. These can include stable isotope analysis, fatty acid analysis, measuring predator and prey population changes over time or space, forensic pathology of prey carcasses, and, of course, predator diet analysis.

Stable isotope and fatty acid analysis reflect the assimilation of prey elements in predator tissues. Using stable isotopes, the trophic level of a predator can be estimated, as well as the primary source of organic matter sustaining a food web (Najera-Hillman et al. 2009; van der Bank et al. 2011). However, this approach requires a complete knowledge of prey isotopic signatures, and these can be difficult to obtain (Corse et al. 2010). Furthermore, isotopic values in prey can overlap, causing a failure to identify specific trophic interactions (Carreon-Martinez and Heath 2010; Hardy et al. 2010). Using fatty acid analysis, prey in a predator's diet can sometimes be determined to species, however, it incorporates a high level of uncertainty that necessitates complicated calibrations and this can sometimes produce inaccurate results (Williams and Buck 2010; Bowles et al. 2011).

Measuring predator and prey population changes over time can accurately reveal predator-prey relationships (see review by Sih et al. 1985; and meta-analysis by Salo et al. 2010). These generally need to be conducted over a long time period, particularly where prey are long-lived and do not necessarily reproduce every year, as is the case for New Zealand’s native frogs (Bell and Wassersug 2003; Bell and Pledger 2010). Furthermore, invasive species are more likely to be generalist predators (Dukes and Mooney 1999), and as such tend to be buffered from fluctuations in the abundance of any one prey species (Inayat et al. 2011). So, native amphibian prey populations would not necessarily be expected to fluctuate in tandem with introduced generalist predators. Another major difficulty with spatially
comparing amphibian abundances, for example at sites with and without predator control (\textit{sensu} Mussett, 2005; Baber \textit{et al}., 2008; Nájera-Hillman \textit{et al}., 2009), is that a difference in abundances may not reflect a difference in population size, but only in detection probability, which can vary spatially or temporally (Crossland \textit{et al}., 2005). This is a major source of bias, particularly given the many life-history and habitat requirement traits that can make amphibians difficult to study (see Chapter 1, Section 1.1 for more on this).

Forensic pathology of prey is useful in cases where predators have a specific preference for prey body parts or leave regular bite marks or other tell-tale signs. For example, Beckmann and Shine (2011) observed that in every case (n = 10) of a raptor ingesting a cane toad (\textit{Rhinella marinus}), raptors had broken and peeled back the lower jaw, and removed the toad’s tongue. Bradford (1991) noted that Brewer's blackbirds (\textit{Euphagus cyanocephalus}) commonly left behind the skeleton and rear limb skin of mountain yellow-legged frogs (\textit{Rana muscosa}). An interesting case was observed in Germany, where a multitude of toads at one pond in Hamburg had apparently ‘exploded’. Pathology reports from Frank Mutschmann (unpublished) revealed that crows had pecked through the toads’ skin in order to specifically remove the liver. These toads use a puffing up response to predators, but with a hole in the toads’ abdomens, this lead to expulsion of their intestines.

I have shown in this thesis, that although exhibiting preference for body parts in some cases, small mammals tended to either ingest whole frogs (removing the potential for forensic pathology) or to choose body parts no more than would be expected by chance, precluding predator identification from frog carcasses (Chapter 2). Forensics can also include the identification of bite marks left on prey carcasses (e.g. Thurley and Bell 1994b; Whitworth \textit{et al}., 2005). In the present study, mice rarely left bite marks and ship rats left them in just over half of cases (Chapter 2), meaning that it would not always be possible to assess bite marks from frog carcasses left by small mammals. Furthermore, the bite marks observed on frog
carnasses were highly variable in size, most likely due to the relatively delicate nature of frog skin, highlighting the degree of caution that must be used when interpreting data from such results.

Diet analysis offers direct evidence of predation and can be used to infer predation rates on native amphibians (see Chapter 5). There are a number of methods through which diet analysis can be carried out. This thesis is primarily concerned with traditional morphological analysis and the emerging field of DNA-based diet analysis. For an overview of other methods used (i.e. enzyme electrophoresis and immunological assays) see Chapter 1, Section 1.5.1.

This is the first time that the detectability of prey DNA has been measured over time in mammal stomach contents and the first time that the detectability of prey DNA has been compared, between stomach and faecal contents, over time in any vertebrate. Until now, the vast majority of laboratory-based studies using DNA-based diet analyses have focused on invertebrate predators and prey (see review by King et al. 2008). I have shown that DNA-based diet analysis not only results in a much higher success rate of detecting amphibians as prey of small mammals, but also greatly extends the period during which detection is possible, exceeding the known gastric emptying and gastrointestinal tract transit times for the predator species studied (see Chapter 4, Table 4.5 for relevant times and references). This has major implications for small mammal diet analysis, as it shows that (at least in some cases) the detection of prey DNA is limited primarily by its presence, rather than the degradative effects of small mammal ingestion or digestion. Many previous studies have used DNA-based diet analysis to investigate field-collected vertebrate stomach and faecal samples (see Section 1.5.1). Where these studies included small mammals, it is possible, given the results presented in this thesis, that the detection of prey DNA may have represented the actual number of prey species present in the samples, rather than some unknown proportion of the
total prey species. This would not have been known to the researchers at the time, but it allows for far greater inferences to be made regarding predation rates and predator diet composition, based on prey detections. Of course, there are many considerations to be taken into account when using DNA-based diet analysis, including methods of sample collection, sample storage, DNA extraction and amplification protocols, and predator and prey characteristics (see Sections 4.4.1 and 5.4.7 for further discussion) and these will include study-specific variations, but the results presented in this thesis show enormous promise for small mammal diet analysis.

The advantages of DNA-based diet analysis are really a combination of the following attributes: 1) Compared to many methods it is inexpensive and involves relatively fewer labour-intensive steps; 2) It is highly accurate and can be used to identify most taxonomic levels (at least to species level); 3) Once suitable primers have been developed, a high throughput of sample processing can be achieved; 4) The skills and equipment required for PCR are now widely available.

However, there are drawbacks to DNA-based diet analysis including: 1) Data based on diet analyses will always only represent the last few meals consumed by the predator, and so may not represent a long-term dietary trend or a predator’s trophic position; 2) Comparative sequencing is a necessary step and relies on a library of prey sequences – if a certain prey species is not in the library, it may be inaccurately identified, or the sequencing of additional potential prey species may be required to be added to the library; 3) Although some recent studies have had some success using quantitative PCR (e.g. Bowles et al. 2011), it is often not possible to accurately estimate how much prey has been consumed (either in terms of biomass or number of prey eaten) – this leads to a reduction in the amount of information that can obtained from each sample.
It seems that DNA-based techniques are currently superseding, or at least being increasingly used in conjunction with, other diet analysis methods and they have proved to be highly effective and versatile. Recently, studies have begun to incorporate morphological analysis, stable isotope analysis and DNA-based analysis in combination and it appears that this approach can offer the most complete overview of the trophic position, food web complexity and diet compositions of the ecosystems and species studied (Carreon-Martinez and Heath 2010; Hardy et al. 2010; Bradford et al. 2013; Maloy et al. 2013). Of course, using multiple methods demands additional resources.

The choice of method used to infer predation will ultimately depend on the question(s) being asked as different methods yield information on different ecological aspects. In many cases, research into the effects of introduced fauna on endangered amphibian prey species will require information on only the direct interactions between a few species of predators and prey. Also, the information is likely to be required urgently so that management and mitigation measures can be put in place. For these reasons, as well as the points made above regarding the various techniques available, it appears that DNA-based diet analysis offers the most reliable, cost-effective and suitable method to aid conservation managers in rapidly assessing the impact of introduced fauna on endangered amphibians worldwide, so that appropriate action can be taken.

6.2 Management Implications and recommendations for endangered amphibians

Mammals are likely to be currently under-represented as predators of frogs worldwide. All the small mammal species presented with southern bell frogs under laboratory conditions ingested them, either wholly or partially (Chapter 2). However, traditional morphological diet analysis was unreliable in detecting frogs as prey (Chapter 3). The review by Toledo et al. (2007) showed that the animal group most reported as preying on post-metamorphic frogs was Reptilia (especially Serpentes; see Chapter 1; Figure 1.1). However, the review was
restricted to reports only where frogs as prey were identified to species level. This may have caused a major bias against the inclusion of mammalian predators, as identification to species level is very difficult for the prey of this group. Furthermore, the detection of native New Zealand frogs in small mammal diets would have gone unobserved using only morphological diet analysis (Chapter 5), and a subsequent estimation of predation rate would have been impossible.

Because the ingestion rate of mammals is directly proportional to their body mass (Pilbeam and Gould 1974; Gould 1975; Druzinsky 1993; Shipley et al. 1994; Gerstner and Gerstein 2008; Chapter 3 of this thesis) and highly correlated with chewing frequency and intensity (Shipley et al. 1994), the under-representation of frogs in mammal stomach or faecal samples may decrease as predator body mass increases. For example, when analysing field-collected scat samples of various mammal species (weighing 0.5 kg to 18 kg), Pinto Llona and Andrews (1999) observed that larger frogs underwent mastication whereas smaller individuals were swallowed whole, preserving the bone structures more completely. However, larger mammal species are sometimes known to consume only the soft parts of amphibians to avoid toxic defences (e.g. European otters Lutra lutra; Lizana and Pérez Mellado, 1990), so this may not always be the case.

Based on the information presented in this thesis, I make the following wildlife management recommendations with respect to amphibian conservation:

1. Where the impact of introduced fauna on native amphibians is unknown, estimates of predation rates should be obtained using diet analysis, as this rapidly provides the information necessary to make decisions regarding management and mitigation of the impacts.
2. Where such diet analyses are being carried out, DNA-based methods should be employed as these offer the most reliable and cost-effective approach.

3. Predation rates provide one integral part of the overall global amphibian decline crisis. However, declines can be caused by multiple factors, sometimes acting synergistically (Alford et al. 2001; Stuart et al. 2004). It is therefore recommended that, once enough data are available, predation rates be incorporated into larger population viability analysis models.

4. New Zealand’s rare and endangered frogs are being negatively affected by introduced mammals. Where feasible, predator removal should be carried out in order to protect native frogs. Ideally, this should be coupled with frog population monitoring pre- and post-predator removal so that recovery of frog populations can be measured.

5. This is the first time that quantifying the impact of ship rats on New Zealand’s frogs through diet analysis has been attempted. However, it remains unclear whether ship rat predation alone threatens native frogs with extinction. It is recommended that further study sites be subject to similar investigations and that studies be extended to more mammal species, so that cost-benefit analyses of predator removal can be accurately carried out.

6.3 **Recommendations for future research**

The major recent advances in molecular techniques, particularly the advent of next-generation sequencing (NGS), have had great significance for DNA-based diet analysis. NGS techniques have already been applied in a number of diet studies (e.g. Clare et al. 2009; Deagle et al. 2009; Bohmann et al. 2011; Murray et al. 2011; Brown et al. 2012; Pompanon et al. 2012 [review]; Boyer et al. 2013). These advances offer the great advantage of using DNA primers that target broad taxonomic groups, followed by parallel sequencing, providing a much more
complete inventory of prey species. Prior to the development of NGS platforms, mixed amplicons of DNA from multiple prey species was necessarily separated through the more labour-intensive process of DNA cloning. So far, NGS has not been applied to the study of the impact of introduced fauna on amphibians. However, group primers have been developed that target the majority of frog species worldwide, and these have been successfully used to detect predation by ship rats on frogs in Australia (see Appendix J for more details). This area of research holds great promise in advancing our understanding of the impact of introduced predators in the greater domain of the global amphibian crisis.

The other major area of predator research pertaining to amphibian conservation is simply the application of the prey DNA detection methods described in this thesis to field-based predation rate studies. If we are to curb the impact of introduced fauna on native amphibians, we need to know what those impacts are. Without this information it is difficult to adequately allocate resources to suitable wildlife management regimes. Applying DNA-based diet analysis, along with well designed stomach and faecal collection protocols, there is major potential to measure and describe the impacts of introduced fauna on the world’s threatened amphibians.

An interesting study for New Zealand’s native frogs could include the recent mouse (Mus musculus) invasion on Maud Island (DOC 2013). Maud Island frog (Leiopelma pakeka) populations have been monitored there since 1983 (Bell and Pledger 2010). If mice prove difficult to eradicate, this could act as a sort of ‘natural experiment’ that could include DNA-based diet analysis and pre- and post-invasion population monitoring to address questions regarding the apparent historical extirpation of this frog species from the mainland. This would also have the potential to explain some of the evolutionary reasons behind why some native frog species have survived on the mainland (in the presence of introduced mammals) while others have not.
Overall, the highly original research detailed in this thesis has provided valid ecological tools to study the impact of introduced mammals on amphibians worldwide that can easily be adapted for any amphibian prey species of concern. As more data become available on the life histories and population dynamics of native frogs, as well as on the predation rates by predators, it is expected that reliable and accurate population viability analyses can be developed to assess the impacts of introduced predators. Once the threats have been identified and assessed in terms of risk management, effective predator control can be implemented, resulting in a decrease in the impacts of introduced fauna and the conservation of the world’s rich, diverse, culturally important, economically beneficial, and very beautiful amphibian biota.


Boulenger GA (1890) The fauna of British India, including Ceylon and Burma: reptilia and batrachia. Taylor & Francis


Clark G (1973) *Staining procedures used by the biological stain commission*. Williams & Wilkins


Crowder MMJ, Hand DJ (1990) *Analysis of repeated measures.* CRC Press


References


de Vos CM (1938b) Studies on the Liopelmidae, No. 2. The inscriptive ribs of Liopelma and their bearing upon the problem of abdominal ribs in vertebrates. Anatomischer Anzeiger 87: 82-101.


References


Emlen JM (1973) *Ecology: an evolutionary approach*. Addison-Wesley, Reading, Massachusetts, USA


Fitzgerald BM, Campbell DJ (2002) Examination of eight *Leiopelma* frogs from Whareorino Forest for signs of predation. Wallaceville, Upper Hutt, Wellington, Ecological Research Associates of New Zealand


References


References


References


References


References


Nájera-Hillman E, King P, Alfaro AC, Breen BB (2009b) Effect of pest-management operations on the abundance and size-frequency distribution of the New Zealand
References


Owen R (1840) *Odontography; or, a treatise on the comparative anatomy of the teeth; their physiological relations, mode of development and microscopic structure, in the vertebrate animals*. Hippolyte Bailliere, London


References


References


van der Bank MG, Utne-Palm AC, Pittman K, Sweetman AK, Richoux NB, Brüchert V, Gibbons MJ (2011) Dietary success of a ‘new’ key fish in an overfished ecosystem:


References


densities of the coqui, *Eleutherodactylus coqui* (Anura: Leptodactylidae) in newly

Worthy TH (1986) *Subfossil bones of the frog Leiopelma in New Zealand*. MSc.

Worthy TH (1987a) Osteology of *Leiopelma* (Amphibia: Leiopelmatidae) and

Worthy TH (1987b) Palaeoecological information concerning members of the frog

Worthy TH, Holdaway RN (1994) Scraps from an owl's table - predator activity as a
significant taphonomic process newly recognised from New Zealand Quaternary
deposits. *Alcheringa* 18(3): 229-245.

Worthy TH, Tennyson AJD, Scofield RP, Hand SJ (2013) Early Miocene fossil frogs

Yoccoz NG, Nichols JD, Boulinier T (2001) Monitoring of biological diversity in

Evaluation of DNA extraction kits for molecular diagnosis of human *Blastocystis*

detection of prey DNA amongst the gut contents of invertebrate predators provide a
new technique for quantifying predation in the field? *Molecular Ecology* 8(12): 2081-
2088.

Zarzoso-Lacoste D, Corse E, Vidal E (2013) Improving PCR detection of prey in
molecular diet studies: importance of group-specific primer set selection and

Zeale MRK, Butlin RK, Barker GLA, Lees DC, Jones G (2010) Taxon-specific PCR
236-244.


Appendices

Appendix A: Known predation events with frogs as prey in New Zealand

Table A-1: Collation of all reported observations of predation events with either native or introduced frogs as prey in New Zealand

<table>
<thead>
<tr>
<th>Prey species</th>
<th>Predator Species</th>
<th>Additional information</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Archey’s frog (<em>Leiopelma archeyi</em>)</td>
<td>ship rat (<em>Rattus rattus</em>)</td>
<td>12 dead frogs with ship rat bite marks evident, plus an additional 5 dead frogs with ship rats as suspected cause of death</td>
<td>Thurley and Bell (1994b), Fitzgerald and Campbell (2002), O. Overdyck (<em>pers. comm.</em>)</td>
</tr>
<tr>
<td>Hochstetter’s frog (<em>Leiopelma hochstetteri</em>)</td>
<td>green and golden bell frog (<em>Litorea aurea</em>)</td>
<td>1 green and golden bell frog stomach inspected and found to contain an Archey’s frog</td>
<td>Thurley and Bell (1994b)</td>
</tr>
<tr>
<td></td>
<td>ship rat</td>
<td>1 dead frog with ship rat bite marks evident</td>
<td>Fitzgerald and Campbell (2002)</td>
</tr>
<tr>
<td></td>
<td>stoat (<em>Mustela erminea</em>)</td>
<td>1 stoat observed carrying live frog, 2 further dead frogs with likely stoat bite marks evident</td>
<td>Baber <em>et al.</em> (2008)</td>
</tr>
<tr>
<td></td>
<td>banded kokopu (<em>Galaxias fasciatus</em>)</td>
<td>1 ingested frog found (out of 369 stomachs)</td>
<td>West <em>et al.</em> (2005)</td>
</tr>
<tr>
<td>Hamilton’s frog (<em>Leiopelma hamiltoni</em>)</td>
<td>tuatara (<em>Sphenodon punctatus</em>)</td>
<td>1 frog hind limb found in tuatara faeces (out of 14 faecal samples)</td>
<td>Newman (1977)</td>
</tr>
<tr>
<td>Prey species</td>
<td>Predator Species</td>
<td>Additional information</td>
<td>References</td>
</tr>
<tr>
<td>------------------------------------</td>
<td>----------------------------------------</td>
<td>----------------------------------------------------------------------------------------</td>
<td>---------------------------------</td>
</tr>
<tr>
<td>Hamilton’s frog or Maud Island frog (<em>Leiopelma pakeka</em>)</td>
<td>laughing owl (<em>Sceloglaux albifacies</em>), extinct</td>
<td>Fossil deposit of prey collected – minimum of 8 individual frogs out of 79 total prey individuals. Not distinguished as separate species at the time of the study</td>
<td>Worthy and Holdaway (1994)</td>
</tr>
<tr>
<td>green and golden bell frog</td>
<td>brown kiwi (<em>Apteryx australis</em>)</td>
<td>Identified in kiwi faeces in an area with a high abundance of frogs, yet the incidence in scats was very low.</td>
<td>Kleinpaste (1990)</td>
</tr>
<tr>
<td></td>
<td>weka (<em>Gallirallus australis</em>)</td>
<td>Frog remains in gizzards (frequency of occurrence 4.7% and 2.9% in respective studies)</td>
<td>Carroll (1963), Coleman et al. (1983)</td>
</tr>
<tr>
<td></td>
<td>feral pig (<em>Sus scrofa</em>)</td>
<td>1 frog (out of 50 stomachs)</td>
<td>B. Egether and C. Krull (unpublished data)</td>
</tr>
<tr>
<td>brown tree frog (<em>Litoria ewingii</em>)</td>
<td>cat (<em>Felis catus</em>)</td>
<td>5 frogs brought to owners’ houses (out of 981 prey)</td>
<td>Morgan et al. (2009)</td>
</tr>
<tr>
<td></td>
<td>ferret (<em>Mustela putorius furo</em>)</td>
<td>1 frog out of 12 stomachs</td>
<td>Clapperton et al. (2011)</td>
</tr>
<tr>
<td>Prey species</td>
<td>Predator Species</td>
<td>Additional information</td>
<td>References</td>
</tr>
<tr>
<td>--------------------------------------</td>
<td>---------------------------</td>
<td>----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------</td>
<td>-----------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>southern bell frog</td>
<td></td>
<td>Both Thurley and Bell (1994b) and Mace (2005) cite Bruce. W. Thomas pers. comm., and Littlejohn (1982) mentions unpublished observations, when stating southern bell frogs are known to kill brown tree frogs in the South Island</td>
<td>Thurley and Bell (1994b), Littlejohn (1982), Mace (2005)</td>
</tr>
<tr>
<td>southern bell frog or brown tree frog</td>
<td>European hedgehog</td>
<td>Present in 15% of hedgehog scats collected (and 31% of scats collected in frog breeding area), Also 3 hedgehogs observed eating live frogs</td>
<td>Brockie (1959), Stewart (2012), Stephen Chadwick (pers. comm.)</td>
</tr>
<tr>
<td>cat</td>
<td></td>
<td>1 frog out of 558 prey items brought to owner’s house (all from 1 cat). Cat seen carrying 1 frog.</td>
<td>Flux (2007), Phil Bishop (pers. comm.)</td>
</tr>
<tr>
<td>southern bell frog or brown tree frog</td>
<td>ferret</td>
<td>In c. 17% of scats, could not be identified to species level.</td>
<td>Roser and Lavers (1976)</td>
</tr>
<tr>
<td></td>
<td>little owl</td>
<td></td>
<td>Marples (1942)</td>
</tr>
<tr>
<td>southern bell frog or green and</td>
<td>little shag</td>
<td>8 frogs in 2 stomachs (from 242 owls)</td>
<td>Dickinson (1951)</td>
</tr>
<tr>
<td>golden bell frog</td>
<td></td>
<td>2 out of 56 stomachs. Not distinguished as separate species at the time of the study</td>
<td></td>
</tr>
</tbody>
</table>
Appendix B: Frog carcasses following small mammal predation

Figure B-1. The carcasses of three frogs (A – C) following removal of body parts by ship rats under laboratory conditions. A1: Ventral view of skull portion, vertebral column and portion of left femur; A2: Ventral view of vertebral column and remains of rear limbs; B1: Dorsal view of head, anterior portion removed; B2: Dorsal view of abdomen, abdominal cavity opened, gastrointestinal tract removed, but not ingested by ship rat; C1: Dorsal view of head, anterior portion removed; C2: Ventral view of head and pectoral region, mandible and associated soft tissue removed. Scales vary and are included on each plate.
Figure B-2. The carcasses of four frogs (D – G) following removal of body parts by ship rats under laboratory conditions. D1: Lateral view of left rear foot with some skin and muscle removed; D2: Dorsal view of pelvic region; E: Dorsal view of right forelimb and right side of abdominal region with some skin and muscle removed; F: Ventral view of anterior abdominal region – an example of skin avoidance - abundant skin remaining, but viscera largely removed; G1: Ventral view of skull and dorsal view of abdomen and rear limbs – example of bone avoidance – almost all muscle and viscera removed with all skeletal components remaining; G2: Ventral view of vertebral column and rear limbs - example of bone avoidance – almost all muscle and viscera removed with all skeletal components remaining. Scales vary and are included on each plate.
Figure B-3  The carcasses of three frogs (H - J) following removal of body parts by mice under laboratory conditions. H1: Dorsal view of abdominal region and rear limb - viscera and some muscle removed; H2: Ventral view of remaining portion of skull, only mandible and maxilla remain; I1: Ventral view of anterior abdominal region and head - majority head removed, forelimbs remain, abdomen heavily masticated; I2: Ventral view posterior abdominal region - some vertebrae removed, majority viscera removed, skin and muscle heavily masticated; J1: Ventral view head and posterior abdomen - left forelimb removed only; J2: Dorsal view head and posterior abdomen - left forelimb removed only. Scales vary and are included on each plate.
Appendix C: Bite marks on frog carcasses

Figure C-1  Examples of ship rat bite marks on frog carcasses on A: Ventral abdomen; B: Lateral abdomen; C: Ventral abdomen; D: Dorsal abdomen; E: Ventral head; F: Ventral abdomen. Scales vary and are included on each plate.
Figure C-2 Examples of ship rat bite marks on frog carcasses: G: Dorsal head; H: Dorsal posterior abdomen; I: Ventral head; J: Dorsal right rear limb; K: Ventral pelvic region; L: Dorsal right rear limb. Scales vary and are included on each plate.
Figure C-3  Hedgehog (M and N), mouse (O and P) and laboratory Norway rat (Q and R) bite marks on frog carcasses on M: Ventral head; N: Dorsal head; O: Ventrolateral abdomen; P: Ventrolateral abdomen; Q: Ventral posterior abdomen and pelvic region and R: Dorsal left rear limb. Scales vary and are included on each plate.
Appendix D: Prey items observed in small mammal stomach contents

Table D-1 Prey items observed in small mammal stomach contents following ingestion of frogs under laboratory conditions. Also included are: Hours since frog ingestion - time at which subject was euthanized post frog ingestion; Taxonomic level – resolution that could be reached based on prey items observed.

<table>
<thead>
<tr>
<th>Species</th>
<th>Hours since frog ingestion</th>
<th>Prey items identified</th>
<th>Taxonomic level</th>
<th>Justification for taxonomic level</th>
</tr>
</thead>
<tbody>
<tr>
<td>laboratory norway rat</td>
<td>3</td>
<td>maxilla</td>
<td>order</td>
<td>possessing row of pleurodont, homodont, similar sized, pedicellate, cone teeth</td>
</tr>
<tr>
<td>laboratory norway rat</td>
<td>9</td>
<td>angulosplenial dentary</td>
<td>none</td>
<td>could not confirm</td>
</tr>
<tr>
<td>ship rat</td>
<td>3</td>
<td>phalange</td>
<td>none</td>
<td>could not confirm</td>
</tr>
<tr>
<td>ship rat</td>
<td>3</td>
<td>phalange</td>
<td>none</td>
<td>could not confirm</td>
</tr>
<tr>
<td>ship rat</td>
<td>6</td>
<td>maxilla</td>
<td>order</td>
<td>possessing row of pleurodont, homodont, similar sized, pedicellate, cone teeth</td>
</tr>
<tr>
<td>hedgehog</td>
<td>1</td>
<td>astragalus calcaneus</td>
<td>genus</td>
<td>ilium with lateral groove</td>
</tr>
<tr>
<td>hedgehog</td>
<td>1</td>
<td>astragalus calcaneus</td>
<td>genus</td>
<td>ilium with lateral groove</td>
</tr>
<tr>
<td></td>
<td></td>
<td>clavicle</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>ilium</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>vertebrae</td>
<td></td>
<td></td>
</tr>
<tr>
<td>hedgehog</td>
<td>1</td>
<td>angulosplenial</td>
<td>species</td>
<td>digital terminal discs present, same width as toe</td>
</tr>
<tr>
<td></td>
<td></td>
<td>astragalus</td>
<td></td>
<td>almost full webbing on rear foot.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>calcaneus</td>
<td></td>
<td>ilium with lateral groove</td>
</tr>
<tr>
<td></td>
<td></td>
<td>full rear foot</td>
<td></td>
<td>fusion boundary evident along</td>
</tr>
<tr>
<td></td>
<td></td>
<td>humerus</td>
<td></td>
<td>entire shaft of radioulna</td>
</tr>
<tr>
<td></td>
<td></td>
<td>maxilla</td>
<td></td>
<td>proximal end of humerus</td>
</tr>
<tr>
<td></td>
<td></td>
<td>radioulna</td>
<td></td>
<td>curved medially.</td>
</tr>
<tr>
<td>hedgehog</td>
<td>3</td>
<td>mandible</td>
<td>order</td>
<td>mentomeckelian bones present, non-dentate</td>
</tr>
<tr>
<td>hedgehog</td>
<td>6</td>
<td>astragalus calcaneus</td>
<td>species</td>
<td>digital terminal discs present, same width as toe</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ilium</td>
<td></td>
<td>almost full webbing on rear foot.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>femur</td>
<td></td>
<td>ilium with lateral groove</td>
</tr>
<tr>
<td></td>
<td></td>
<td>full rear foot</td>
<td></td>
<td>fusion boundary evident along</td>
</tr>
<tr>
<td></td>
<td></td>
<td>mandible</td>
<td></td>
<td>entire shaft of radioulna</td>
</tr>
<tr>
<td></td>
<td></td>
<td>maxilla</td>
<td></td>
<td>proximal end of humerus</td>
</tr>
<tr>
<td></td>
<td></td>
<td>vertebrae</td>
<td></td>
<td>curved medially.</td>
</tr>
</tbody>
</table>
Appendix E: Prey items observed in small mammal faecal contents

Table E-1 Prey items observed in small mammal faecal contents following ingestion of frogs under laboratory conditions. Also included are: Hours since frog ingestion - time at which faeces were collected post frog ingestion; Taxonomic level – resolution that could be reached based on prey items observed.

<table>
<thead>
<tr>
<th>Species</th>
<th>Hours since frog ingestion</th>
<th>Prey items identified</th>
<th>Taxonomic level</th>
<th>Justification for taxonomic level</th>
</tr>
</thead>
<tbody>
<tr>
<td>ship rat</td>
<td>6</td>
<td>phalange</td>
<td>none</td>
<td>could not confirm</td>
</tr>
<tr>
<td>ship rat</td>
<td>12</td>
<td>phalange</td>
<td>none</td>
<td>could not confirm</td>
</tr>
<tr>
<td>ship rat</td>
<td>18</td>
<td>phalange</td>
<td>none</td>
<td>could not confirm</td>
</tr>
<tr>
<td>hedgehog</td>
<td>6</td>
<td>clavicle</td>
<td>genus</td>
<td>ilium with lateral groove</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ilium</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>phalanges</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>tibiofibula</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>vertebrae</td>
<td></td>
<td></td>
</tr>
<tr>
<td>hedgehog</td>
<td>6</td>
<td>ilium</td>
<td>genus</td>
<td>femur not distinctively sigmoid,</td>
</tr>
<tr>
<td></td>
<td></td>
<td>humerus</td>
<td></td>
<td>and lacking strong femoral crest</td>
</tr>
<tr>
<td></td>
<td></td>
<td>femur</td>
<td></td>
<td>ilium with lateral groove</td>
</tr>
<tr>
<td></td>
<td></td>
<td>phalanges</td>
<td></td>
<td>fusion boundary evident along</td>
</tr>
<tr>
<td></td>
<td></td>
<td>radioulna</td>
<td></td>
<td>entire shaft of radioulna</td>
</tr>
<tr>
<td></td>
<td></td>
<td>tibiofibula</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>vertebrae</td>
<td></td>
<td></td>
</tr>
<tr>
<td>hedgehog</td>
<td>6</td>
<td>radioulna</td>
<td>genus</td>
<td>fusion boundary evident along</td>
</tr>
<tr>
<td></td>
<td></td>
<td>vertebrae</td>
<td></td>
<td>entire shaft of radioulna</td>
</tr>
<tr>
<td>hedgehog</td>
<td>6</td>
<td>sternum</td>
<td>order</td>
<td>fusion of radius and ulna</td>
</tr>
<tr>
<td></td>
<td></td>
<td>radioulna</td>
<td></td>
<td>unique to anura</td>
</tr>
<tr>
<td>hedgehog</td>
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<td>angulosplenial</td>
<td>genus</td>
<td>femur not distinctively sigmoid,</td>
</tr>
<tr>
<td></td>
<td></td>
<td>astragalus</td>
<td></td>
<td>and lacking strong femoral crest</td>
</tr>
<tr>
<td></td>
<td></td>
<td>calcaneus</td>
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<td>ilium with lateral groove</td>
</tr>
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<td></td>
<td></td>
<td>femur</td>
<td></td>
<td>fusion boundary evident along</td>
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<td></td>
<td></td>
<td>phalanges</td>
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<td>entire shaft of radioulna</td>
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<td></td>
<td></td>
<td>radioulna</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>vertebrae</td>
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<td></td>
</tr>
<tr>
<td>hedgehog</td>
<td>6</td>
<td>coracoid</td>
<td>genus</td>
<td>ilium with lateral groove</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ilium</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
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Appendix F: Representative photographs of prey items in stomach and faecal contents

Figure F-1 Examples of frog remains found in small mammal stomach contents (item, mammal species, h since frog ingestion). A: Full rear foot, hedgehog, 1 h; B: Maxilla, laboratory Norway rat, 1 h; C: Ilium, hedgehog, 1 h; D: Radioulna, hedgehog, 1 h; E: Clavicle, hedgehog, 1 h; F: Astragalus and calcaneum, hedgehog, 1 h.
Figure F-2  Examples of frog remains found in small mammal stomach contents (item, mammal species, h since frog ingestion). For comparison *L. raniformis* specimen bones are also shown (I – L).  

- **G**: Ilia, hedgehog, 6 h  
- **H**: Maxilla, ship rat, 6 h  
- **I**: Clavicles (above) and coracoids (below);  
- **J**: Astragalus and calcaneum;  
- **K**: Ilium;  
- **L**: Maxillae (above) and humeri (below).
Figure F-3  Examples of bone fragments observed in small mammal stomach contents that could not be identified (potential origin of fragment; mammal species; h since frog ingestion).  A: Skull, ship rat, 1 h;  B: Dentary and angulosplenial, laboratory Norway rat, 9 h;  C: Phalange, ship rat, 6 h;  D: Urostyle, laboratory Norway rat, 3 h;  E: Mandible, hedgehog, 3 h;  F: General bone shards, laboratory Norway rat, 3 h.
Figure F-4 Examples of frog remains found in small mammal faecal contents (item, mammal species, h since frog ingestion). A: Radioulna, hedgehog, 12 h; B: Femurs, hedgehog, 6 h; C: Broken tibiofibula, hedgehog, 6 h; D: Bone shards, unidentified, laboratory Norway rat, 24 h; E: Bone shards, unidentified, laboratory Norway rat, 12 h; F: Bone fragment, unidentified, ship rat, 18 h.
Appendix G: GLM results – Ingestion, digestion and prey bone length

Details of general linear models used to examine the relationship between ingestion rate, digestion and prey bone length in small mammal stomach and faecal samples

Time as a continuous factor:

Using the stomach sample data set, the model with the lowest AIC value (following stepAIC) was a full factorial model with all the relevant factors included. According to this model none of the factors or interactions among factors significantly affected mean maximum prey bone length ($p > 0.1$ for all factors), indicating that maximum prey bone length did not decrease over time.

Model: glm(PZbonesize ~ species * PZhrs * PZgmin);
PZbonesize – maximum prey bone length
PZhrs – hours since prey ingestion
PZgmin – ingestion rate

Coefficients:

|                | Estimate | Std. Error | t value | Pr(>|t|) |
|----------------|----------|------------|---------|----------|
| (Intercept)    | -0.03837 | 0.62656    | -0.061  | 0.952    |
| species2       | -0.93003 | 0.99639    | -0.933  | 0.362    |
| species4       | 1.31006  | 3.60245    | 0.364   | 0.720    |
| PZhrs          | 0.12630  | 0.50101    | 0.252   | 0.804    |
| PZgmin         | 0.05700  | 1.03820    | 0.055   | 0.957    |
| species2:PZhrs | 0.52338  | 0.93544    | 0.559   | 0.582    |
| species4:PZhrs | 1.32656  | 3.21814    | 0.412   | 0.685    |
| species2:PZgmin| 1.31267  | 1.45471    | 0.902   | 0.378    |
| species4:PZgmin| 0.87867  | 2.92066    | 0.301   | 0.767    |
| PZhrs:PZgmin   | 0.04608  | 0.83343    | 0.055   | 0.956    |
| species2:PZhrs:PZgmin | -7.91694 | 5.18425    | -1.527  | 0.143    |
| species4:PZhrs:PZgmin | 1.61892  | 2.62395    | 0.617   | 0.545    |

For the faecal sample data set, the model that fitted the data best according to AIC values included species and time since ingestion. According to this model time since prey ingestion approaches, but doesn’t quite reach, significance ($t = -2.22$, df = 8, $p = 0.057$).
Appendices

Model: `lme(PZFbonesize ~ PZFhr+Fspecies, random=~1|Fid2, method="ML")`

**PZFbonesize** - maximum prey bone length

**PZFhr** – hours since prey ingestion

**Fid2** – subject ID

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**Time as a categorical factor:**

Using the stomach sample data set, none of the factors helped to account for the variance in mean maximum bone length, and the null model resulted in the lowest AIC value, again indicating that maximum prey bone length did not change over time.

`glm(PZbonesize ~ 1)`

Coefficients:

|           | Estimate | Std. Error | t value | Pr(>|t|) |
|-----------|----------|------------|---------|----------|
| (Intercept)| -7.931e-16 | 1.796e-01 | 0       | 1        |

For the faecal sample data set, the model that best fitted the data according to AIC values included only species as a factor, again indicating that time did not have a significant effect.

`lme(PZFbonesize ~ Fspecies, random=~1|Fid2, method="ML")`

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Appendix H: 12S sequences for *Litoria ewingii* and *Litoria raniformis*

**Sequence H-1** Partial sequence of mtDNA (12S ribosomal RNA gene) for *Litoria ewingii*, fragment amplified using general 12S primers described by Kocher *et al.* (1989).

*Litoria ewingii*

CTATGTCTAGTGTAATTTAAATTACACCCTAATGCTGGGAATCTACGAGCAAGCTTAAACCCAAAGGACTGGAATCTGGCTCCACGTTACTCCCTCAATCGATCCCTTATTATGCTGCTGACCTCTCAATACGGTTACGCGTCTTTT

**Sequence H-2** Partial sequence of mtDNA (12S ribosomal RNA gene) for *Litoria raniformis*, fragment amplified using general 12S primers described by Kocher *et al.* (1989).

*Litoria raniformis*

CAACTGGGATTAGATACCCCACTATGCCCGTAAATTTAAATTACACCCTAATGCTGGGAATCTACGAGCAAGCTTAAACCCAAAGGACTGGAATCTGGCTCCACGTTACTCCCTCAATCGATCCCTTATTATGCTGCTGACCTCTCAATACGGTTACGCGTCTTTT

Appendix I: Alignment showing all mainland frog species and amplified prey DNA

Alignment I-1 Example alignment of partial 12S mtDNA using *Litoria raniformis* as the heading reference. Corresponding sequences for all other mainland New Zealand frog species are also shown. Also included are amplified fragments (using the primer pair 12S-LR-F / 12S-LR-R detailed in Section 4.2.4) from prey in the stomach contents of two subjects (laboratory Norway rat 1 and Hedgehog 3) that had ingested frogs under laboratory conditions.

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Appendix J: Report on group-specific approach to detect frogs as prey

Title

Analysis of mammalian stomach and faecal samples from Australia for the presence of anuran prey using a DNA-based group primer approach

Introduction

Fox, cat, ship rat and mouse diet samples (n = 97) were obtained from Mr. James Garnham, University of Newcastle, Australia, along with tissue samples from the 6 species of frogs (*Litoria fallax*, *L. jervisiensis*, *L. peronii*, *Limnodynastes peronii*, *Lim. dumerilii* and *Crinia signifera*) known to be present on the sites from which mammal diet samples were collected.

This report describes the procedures used to assay for the presence of anuran DNA in the diet samples, carried out by Mr. Bastian Egeter at the University of Otago, New Zealand.

Method

Samples were homogenised using a rotor-stator homogeniser (IKA Ultra-Turrax T25; IKA-Labortechnik) with a 10 mm diameter plastic homogenising probe (S 25 D - 10 G – KS; IKA-Labortechnik) at 8,000 RPM. DNA was then extracted using either the ‘Qiagen DNeasy blood and tissue’ kit (Qiagen; stomach contents; n = 85) or the ‘Zymo D6010 Faecal DNA’ kit (Zymo Research; faecal contents; n = 12) following the manufacturers’ instructions. PCRs were performed on the extracted DNA, using degenerate taxon-specific primers that were designed to target a section of the mtDNA gene coding for 16S rRNA (Bastian Egeter, unpublished). These primers were developed with the aim of amplifying the DNA from all, or most, frog species around the world, but not amplifying DNA from any other taxa. The primer pair used was NON-3:
The PCR procedure was as follows: 1 µl DNA (c. 50 ng) in 10 µl of reagent mix; 1 x NH4 buffer (BIOLINE), 1.5 mM MgCl2 (BIOLINE), 0.2 mM dNTPs, 0.5 µM of each primer (forward and reverse), 0.5 U BIOTAQ (BIOLINE). The thermal cycling profile was: initial step for 2 min at 94 °C; then 30 cycles of the following: denaturation at 94 °C for 15 s, annealing at 49 °C for 25 s, and extension at 72 °C for 30 s (using the Eppendorf Mastercycler Pro 6321 PCR machine). Thermal cycling conditions were: 94 °C for 2 minutes, then 30 cycles (Step 1: 94 °C for 15 s / Step 2: 49 °C for 25 s / Step 3: 72 °C for 30 s). Aerosol-resistant pipette tips were used throughout all PCR procedures. PCR products were separated on 2 % agarose (BIO-41025, Bioline) gels containing 2 X fluorescent nucleic acid stain (SYBR safe DNA gel stain, Invitrogen) and allowed to run through gel electrophoresis for 30 min at 100 V. Fluorescent DNA bands were subsequently viewed with a blue light transilluminator. All positive PCRs were re-run and amplicons were sequenced using the ABI 3730xl DNA Analyser (Applied Biosystems).

All anuran tissue samples were subject to the same procedure (using the 'Qiagen DNeasy blood and tissue' kit; homogenisation step was not necessary) to ensure that the NON 3 primer pair would amplify DNA from all species. DNA extraction from these samples was carried out after all stomach and faecal samples had already been subject to PCR and subsequent sequencing. As there had never been any tissue from these anuran species in the laboratory before, there was no chance that mammalian diet samples could have been contaminated with DNA from these species.

Two approaches were used to assign sequences resulting from mammal stomach and faecal samples to their respective prey species, both using the BLAST® tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi), the BLASTN 2.2.28 program (Zhang et al. 2000) and the MegaBLAST algorithm (Morgulis et al. 2008). Firstly, using the “Align Sequences
Nucleotide BLAST™ option (blastn suite), the unknown prey sequence was entered as the query to be aligned against known 16S gene sequences from the 6 anuran species. These sequences were obtained from the Genbank® (Benson et al. 2010) nucleotide database available from the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/; Accession numbers: FJ945397.1, DQ283245.1, DQ116857.1, EU443926.1, DQ283286.1, DQ116860.1). However, only partial sequences for the 16S gene were available for Lit. fallax, Lit. peronii or Lit. jerviensis, so amplicons from tissue samples of these species were also sequenced and included in the alignments.

Secondly, the unknown prey sequence (i.e. that amplified from predator stomach or faecal contents) was entered as a query using the “Standard Nucleotide BLAST” (blastn suite), without limiting the alignments to any species in particular, but using the entire nucleotide database. The species of prey to which a sequence belonged was identified based on the BLAST® score, query cover, associated E-values, and identities of the alignment results.

**Results**

The NON-3 primer pair successfully amplified DNA from all 6 anuran tissue samples, with the expected amplicon base pair length (232 bp).

Prey DNA was amplified from two of the samples, both from ship rats (*Rattus rattus*) trapped in the “Brickpit” study site on the 10/09/2012. Limiting the BLAST queries to the 6 known anuran species, one sequence was identified as belonging to *C. signifera*, while the other did not return any significant alignments (Table J-1). Extending the alignment queries to the entire nucleotide database produced concurring results for the former sequence and identified the closest match of the latter as belonging to the Eastern water skink (*Eulamprus quoyii*; Table J-1).
The large differences in E-values (the number of BLAST hits that can be expected by chance, given the query sequence and database size), and the percentage of identical bases, between the most significant alignment and the closest alternative species indicates a high level of confidence assigning the sequences to these species (Table J-1).
Table J-1  Results of BLAST alignments for prey DNA sequences amplified in this study.

<table>
<thead>
<tr>
<th>Predator ID</th>
<th>Reference database</th>
<th>Alignment hits</th>
<th>Prey species</th>
<th>BLAST score</th>
<th>Query cover</th>
<th>E-value</th>
<th>Percentage identical bases</th>
<th>Genbank accession number</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>R. rattus</em> T1-4 (2)</td>
<td>6 anuran species (see text)</td>
<td>one significant alignment only</td>
<td><em>Crinia signifera</em></td>
<td>235</td>
<td>91%</td>
<td>4 e^-66</td>
<td>94%</td>
<td>EU443926.1</td>
</tr>
<tr>
<td>nucleotide</td>
<td></td>
<td>most significant alignment</td>
<td><em>Crinia signifera</em></td>
<td>265</td>
<td>91%</td>
<td>7 e^-67</td>
<td>97%</td>
<td>EU443872.1</td>
</tr>
<tr>
<td>nucleotide</td>
<td></td>
<td>closest alternative species</td>
<td><em>Crinia riparia</em></td>
<td>195</td>
<td>91%</td>
<td>1 e^-46</td>
<td>89%</td>
<td>EU443857.1</td>
</tr>
<tr>
<td><em>R. rattus</em> T5 4 (2)</td>
<td>6 anuran species (see text)</td>
<td>no significant similarity found</td>
<td><em>Eulamprus quoyii</em></td>
<td>119</td>
<td>94%</td>
<td>8 e^-24</td>
<td>92%</td>
<td>AF530220.1</td>
</tr>
<tr>
<td>nucleotide</td>
<td></td>
<td>one significant alignment only</td>
<td><em>Eulamprus quoyii</em></td>
<td>136</td>
<td>65%</td>
<td>5 e^-29</td>
<td>85%</td>
<td>AF530220.1</td>
</tr>
<tr>
<td>nucleotide</td>
<td></td>
<td>most significant alignment</td>
<td><em>Eulamprus quoyii</em></td>
<td>100</td>
<td>65%</td>
<td>3 e^-18</td>
<td>78%</td>
<td>KC575645.1</td>
</tr>
<tr>
<td>nucleotide</td>
<td></td>
<td>closest alternative species</td>
<td><em>Eulamprus tympanum</em></td>
<td>100</td>
<td>65%</td>
<td>3 e^-18</td>
<td>78%</td>
<td>KC575645.1</td>
</tr>
</tbody>
</table>

1 Although only one significant alignment was obtained using the MegaBLAST algorithm, a similar alignment was performed using a discontiguous MegaBLAST (optimised for more dissimilar sequences), for the sake of comparison against the closest alternative species.
Conclusions

This study identified two prey items in ship rat diet samples, which were not observed during a prior investigation of these diet samples using traditional morphological analysis (James Garnham, pers. comm.). Ship rats have been observed to meticulously avoid skeletal material when ingesting frogs as prey (Egeter et al. 2011) and such feeding behaviour may be the reason that DNA-based methods detected the prey where morphological methods did not.

The fact that DNA from a reptile *Eulamprus quoyii* (Order: Squamata) was amplified in this study shows that the primers used are not specific to anuran DNA. Here, this characteristic yielded the identification of an additional prey species, which was of some research value. However, there may be cases where this will prohibit the use of these primers (at least using the current PCR protocol). An example of such a case would be the investigation of lizard predation on anuran species, where these primers would be expected to amplify the predator, rather than the prey DNA. Carrying out further PCR optimization steps may result in the primer pairs becoming more specific to their desired targets, and more research is required to validate this.
Appendix K: Possums and ship rats visiting bait tubes

Figure K-1 Photographic evidence of possums (A) and ship rats (B) visiting bait tubes at Whareorino Forest, captured by a motion sensing camera (Bushnell Trophy XLT 119455).
Appendix L: BLAST alignments for field samples testing positive for frogs as prey.

Table L-1. BLAST alignments for field samples testing positive for the presence of frogs as prey. Alignments included corresponding 12S gene sequences for all mainland frog species in New Zealand.

<table>
<thead>
<tr>
<th>Sample details</th>
<th>Alignment hits</th>
<th>Prey species</th>
<th>BLAST score</th>
<th>Query cover</th>
<th>E-value</th>
<th>Percentage identical bases</th>
</tr>
</thead>
<tbody>
<tr>
<td>Waitakere Ranges, Site 3, ship rat, stomach</td>
<td>one significant alignment only</td>
<td><em>L. hochstetteri</em></td>
<td>165</td>
<td>81</td>
<td>$2 \cdot e^{-45}$</td>
<td>97</td>
</tr>
<tr>
<td>Wharerino Forest, Site 2, ship rat, stomach</td>
<td>one significant alignment only</td>
<td><em>L. archeyi</em></td>
<td>154</td>
<td>83</td>
<td>$3 \cdot e^{-42}$</td>
<td>98</td>
</tr>
<tr>
<td>Wharerino Forest, Site 1, ship rat, stomach</td>
<td>one significant alignment only</td>
<td><em>L. archeyi</em></td>
<td>145</td>
<td>78</td>
<td>$2 \cdot e^{-39}$</td>
<td>100</td>
</tr>
<tr>
<td>Omakau, Site 6, hedgehog, stomach</td>
<td>two significant alignments</td>
<td><em>L. raniformis</em></td>
<td>134</td>
<td>93</td>
<td>$1 \cdot e^{-26}$</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>L. aurea</em></td>
<td>121</td>
<td>92</td>
<td>$2 \cdot e^{-32}$</td>
<td>97</td>
</tr>
<tr>
<td>Omakau, Site 6, hedgehog, faeces</td>
<td>three significant alignments</td>
<td><em>L. raniformis</em></td>
<td>300</td>
<td>97</td>
<td>$2 \cdot e^{-86}$</td>
<td>99</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>L. aurea</em></td>
<td>278</td>
<td>97</td>
<td>$3 \cdot e^{-78}$</td>
<td>97</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>L. ewingii</em></td>
<td>250</td>
<td>97</td>
<td>$2 \cdot e^{-71}$</td>
<td>94</td>
</tr>
</tbody>
</table>

* Considered the most likely match given the associated scores and percentage identical bases