Diseases of New Zealand Native Frogs

Thesis submitted by

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MANZCVS Medicine of Zoo Animals

October, 2012

For the degree of Doctor of Philosophy

in the School of Public Health, Tropical Medicine and Rehabilitation Sciences

James Cook University

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Acknowledgements

The reason for starting a PhD would be different for everyone. For me it was about achieving a dream. Proving to myself I could do it. My motto has been "Whatever you believe you can achieve". However, in the valleys of life and this project, I often borrowed the mantra of Dory, the angelfish from Nemo, "Just keep swimming". That phrase has kept me from drowning in the sea of research many a time.

It's obvious no one can do something this large alone and I am no exception. I have certainly learned the art of collaboration and asking for help! So, here goes the long list of thanks.

First and foremost I thank my husband and friend, Todd, for standing by me while I have taken on this mental marathon. It has not been short and sweet and things have always taken longer than I thought. You have assisted me by moving house, starting new businesses, extra childcare duty, making new friends, making lots of dinners and taking on extra duties – usually without reminding me that was all my idea….. Of course my children have been there right alongside him with adjustments: moving to a new country, changing schools, changing cultures and wondering if anyone was going to pick them up from school when I was late from "just finishing something important". Although they may not have understood fully what I was doing, they knew it was important to me and have been there to hug me when I needed it.

My supervisors Rick Speare, Lee Berger, Lee Skerratt and Phil Bishop are a fantastic team and I couldn't have asked for better. It is their combined contributions that have helped me to succeed.

Rick, you have shown me what true humility and generosity is as you live it every day. Even though you travel the world, you have somehow fit me into your busy schedule. When you are with me you are focused and efficient and I feel like this project is as important to you as it is to me. You

have become not only my supervisor but my adopted father, my counselor and my colleague. I continue to hope that at the end of this I am a little more like you.

Lee Berger, you are our local guardian angel and my adopted sister. You have made sure we have had all the necessities to live while settling in Townsville and made the difficult process bearable. The mental support you give has been a lifeline. If I felt hopeless or frustrated you have been there to guide me and to remind me it's all part of the process and that the finished product is always better- and you were right every time. Your reviewing skills are phenomenal and I owe you for my major improvement in scientific writing. I'm still not as good as you, but I am getting there!

Lee Skerratt, you are a great friend to the whole family and my adopted brother. You have also been my necessary devil's advocate! I could always count on you to give me the sometimes not so good honest truth while offering suggestions on improvement. Your practical lessons on experimental design, statistics, manuscript order, presentations, posters, author order, and journal impact factors have been essential and most appreciated.

Phil, you are a great inspiration. You have been an amazing supporter and calm in the face of my mental storms. Your enthusiastic attitude towards frog conservation has infected me and made me into an unabashed frog doctor- one thing I vowed not to become! Your endless circle of students has also provided me with enough competition to keep things moving and to try to finish first- something I haven't always achieved but it provided great impetus. I thank you for all your assistance in New Zealand – I truly could not have achieved what I did without you.

I also thank the fantastic team at the New Zealand Centre for Conservation Medicine at Auckland Zoo while I was there learning to be a zoo vet and to care for native frogs. Firstly is Richard Jakob-Hoff. I would not have started the medicine residency at the zoo nor the PhD without his encouragement and vision. Richard, you also have been an amazing example of what kind of person I want to become. You are a humble achiever who takes the time to get to know everyone and make

them feel special. I thank you for my personal life coaching over my four years with you. I hope I have made you proud.

Thanks to John Potter for allowing me to take over the native frog health programme. You paved the way for the care of these native frogs and your insight and opinions were always valuable. Thanks to Craig Pritchard, manager of the NZCCM, as your support and seemingly endless encouragement for this project was essential to its success. To all the wonderful vet nurses including Lauren Best, Mel Farrant and Nicole Czerinak at the NZCCM who helped me organize treatments, X-rays, and general care for any sick frog - thanks for letting me lean on you as I couldn't do it alone. Special thanks to Kirsten Derry who gave me office support and assistance to the boring bits of this project. Your infectious attitude and smiles kept me going many a day.

Thanks to all the Auckland Zoo native frog keepers and curators: Andrew Nelson, John Rowden, Michelle Whybrow, Tanya Shennan, Natalie Clark, Ian Fraser, Richard Gibson and many more. I learned so much from all of you and your absolute commitment to the frogs' health was inspirational! I am sure asking you to spend hours of your day searching for slaters and hoppers was not your idea of fun- but you did it for the frogs. A very special mention to Peter West and Nicole Kunzmann who absolutely were my right hand in the veterinary care of the frogs. Thanks for all your ideas, discussions (over and over), and willingness to listen to my thoughts and act upon them, with respect, passion and enthusiasm. Your contribution to the native frogs lives within this thesis.

The cooperation and support of the native frog team at the New Zealand Department of Conservation was vital. Avi Holzapfel and Kate McInnes were the impetus in the DOC team to start a PhD on diseases of New Zealand native frogs. It was such a monumental task that I would not have attempted it without their initial backing. The rest of the DOC native frog team including Amanda Haigh, Lisa Daglish, Oliver Overdyk, Dave Smith, and Paul Gasson continued to support disease research when Avi changed positions and have collaborated and assisted me at every step. I know the

processes were sometimes cumbersome and frustrating, but you really put in the effort to get this final project done. I hope we can continue to work together.

Thanks to Ben Bell for being the true patriarch of *Leiopelma* biology in New Zealand. Your quiet humble manner and all the amazing knowledge you have has made you such a pleasure to collaborate with.

Thanks to Dianne Brunton from Massey University, Albany. You are the most amazing professional woman I have met is science and were a huge inspiration to me that I could do this project with a family. I truly thank you for all your guidance.

Thanks to the frog girls at the University of Otago for making me part of the team: Jen Germano - my Dunedin hostess; Joelene Oldham for frog care; Sarah Herbert for her lovely assistance and collaboration; Sabine Melzer and Michel Ohmer for their support.

To the massive amazing team at Landcare Research, Auckland, who took me in for almost a year and made me a part of the team. So many of you spent so much time training me and offering advice on your areas of expertise with such passion – it was a great experience! To name just a few: Zeng Zhao, Bevan Weir, Paula Wilke, Dianne Gleeson, Frank Molinia, Baxter Massey, Daniel Than, Maureen Fletcher, Stanley Bellgard, and of course, Sarah Dodd.

Thanks to the many other collaborating institutions and to all those that took such an interest in native frogs: Brian Gill of The Auckland Museum; Gillian Stone and Colin Miskelly of Te Papa Museum; Hilary Holloway, Maureen Watson and Karen Callon from the University of Auckland; Maurice Alley and Brett Gartrell of the Massey University Veterinary School.

Thanks to Richard Norman and Bruce Waldman for being the pioneers of native frog disease research in New Zealand and enabling me to stand on your shoulders.

Finally, many thanks to all the team at James Cook University from the Amphibian Disease Ecology Group; Anton Breinl Centre; and the School of Public Health, Tropical Medicine and Rehabilitation Sciences. To name a few: Ari Gardner and Sandra Burrows (for answering all my questions and helping me with a smile); Anne-Marie Hill (travel extraordinaire); Kerrianne Watt (your career advice and encouragement were indispensable); Marcia Croucher and Judy Woosnam (for helping me in my paperwork crises); Rebecca Webb, Diana Mendez, Stephen Garland, Sara Bell, Jamie Voyles, Sam Young and Andrew Woodward: you are the best frog team ever and I thank all of you for imparting your frog knowledge to me! My fellow postgrad students Zamir, Vahan, Gail, Felicity, Robin, Michael and Kenji, thanks for listening to my life story and being there to help with those little things that we students go through together. Last, but not least, huge appreciation to the talents of Tricia Emmanuel, who has likely read every word of this thesis as many times as me.

If I have missed anyone, (and you know who you are), huge apologies for the omission. I hoped I thanked you along the way.

Remember, “Whatever you believe you can achieve”, but if that doesn’t work, “Just keep swimming”.

Stephanie D. Shaw

Sept 25th, 2012

Abstract

The aims of this project were to evaluate the health status of New Zealand native frogs (*Leiopelma* spp.) and to investigate what diseases, if any, were limiting their survival both in captivity and in the wild. Issues for captive frogs included nutritional and infectious diseases. For wild frogs I investigated the occurrence of declines and conducted mapping and experimental studies on chytridiomycosis.

Mortality rates and causes of death were analysed for 252 wild-caught *Leiopelma* spp. that were held in captivity in a research program at the University of Canterbury and later transferred to other institutions between 2000 and 2006. *Leiopelma archeyi* and *Leiopelma hochstetteri* had similar overall average mortality by year (12.4% and 14.9% respectively) but different yearly mortality patterns, whereas *Leiopelma pakeka* had much lower overall mortality (3.5%).

On further investigation, metabolic bone disease (MBD) was diagnosed in *L. archeyi* and *L. hochstetteri* in 2008 at three institutions: Auckland Zoo, Hamilton Zoo, and the University of Otago. Radiographs on archived and live frogs showed that MBD had been present at Canterbury, but at a lower rate (3%) than in the current institutions (38-67%). Micro-computed tomography showed that the femoral diaphyses of the captive frogs at Auckland Zoo had greater bone volume, bone surface, cross-sectional thickness and mean total cross-sectional bone perimeter which was consistent with osteofluorosis. On histology of the same femurs there was hyperplasia, periosteal growth, and thickening of trabeculae which was also consistent with skeletal fluorosis. An increase in fluoride levels in the water supply preceded the rise in the incidence of the above pathology further supporting the diagnosis of osteofluorosis. To determine the natural diet of *Leiopelma* spp., stomach contents of sixteen *L. archeyi* from the Coromandel and nine *L. hochstetteri* from the Coromandel, the Hunua Ranges and Maungatautari were analysed. Both species ate a wide range of invertebrates including springtails, mites, ants, parasitic wasps, amphipods and isopods, while *L. archeyi* also ate snails. The

mean ratio of maximum prey size ingested to snout-vent length in *L. archeyi* was 0.31(range 0.16 – 0.5), and in *L. hochstetteri* was 0.42 (range 0.21– 0.75). Analysis of long-standing husbandry practices showed that ultraviolet-B exposure and the dietary calcium:phosphorus ratio was deficient when compared with wild conditions – likely attributing to chronic underlying MBD.

Two novel nematodes (*Koerneria* sp. and *Rhabditis* sp.) were found separately in four captive Archey's frogs showing clinical signs of haemorrhagic purulent nasal discharge and weight loss. One of these frogs also had a novel protozoal infection (*Tetrahymena*) in the nasal cavity. One frog was treated successfully with oral moxidectin at 0.4 mg/kg for the nematode infection and topical metronidazole at 10 mg/kg for the protozoal infection. The clinical signs abated only after both infections were cleared.

Multifocal small domed lesions occurred extensively on the ventral skin of captive *Leiopelma archeyi* at two institutions between 2000 and 2012. Incidence was 41% (34/83) of frogs at Auckland Zoo and 9% (1/11) at the University of Otago and were not linked with an increased risk of death. The lesions had the gross and microscopic characteristics of adenomatous hyperplasia (AH) of the dermal mucous glands which are widely distributed over the skin of normal Archey's frogs. In affected frogs the size and location of lesions varied over time, even resolved completely in some animals, and sometimes reappeared. Histologically the lesions were composed of enlarged mucous glands that expanded the dermis and elevated the epidermis. They were semi-organized, with occasional acinar structures with central lumina sometimes containing mucus. Nuclei had moderate anisokaryosis and mitotic figures were uncommon. The aetiology of this adenomatous hyperplasia is unknown, but factors associated with the captive environment are most likely.

Surveys were distributed to New Zealand land users in 1998 and 2008 to acquire information about the distribution and population levels of both native (*Leiopelma* spp.) and non-native *(Litoria* spp.) frogs. Overall frog populations in New Zealand were reported as declining, but were stable or

increasing in a few regions. Possible causes reported for declines were disease (chytridiomycosis), increase in agriculture and an increase in the distribution of predatory fish.

The current distribution, host species and prevalence where known of the amphibian chytrid fungus *Batrachochytrium dendrobatidis* (Bd) is reported in New Zealand. I conducted histology and PCR on new and archived specimen and also collated previous test records. The data included all regions in New Zealand and six off shore islands at 135 sites with 704 records from over eleven contributors spanning collection dates 1930-2010. The earliest case was from 1999 and we report 132 positive individuals from 54 widespread sites. Bd was detected in all three non-native *Litoria* spp. in five out of sixteen regions but not in the six off-shore areas tested. Bd was not detected in native *L. hochstetteri*, *L. hamiltoni* and *L. pakeka*. Included in the data is a museum survey of 152 individuals from five species from 1930-1999 using histology and Bd specific immunohistochemistry. All museum specimens were negative. In *L. archeyi* at a study site in the Coromandel Ranges, the prevalence of Bd from 2006-2010 was relatively stable at 14-18%. The prevalence of Bd in Whareorino has remained both consistent and low (<50% for the 95% confidence interval upper limit) between 2005-2010.

An experiment infection trial revealed *L. archeyi* may be innately resistant to chytridiomycosis. Six wild-caught *L. archeyi* that naturally cleared infections with Bd while in captivity were exposed again to Bd to assess their immunity. All six *L. archeyi* became reinfected at low intensities, but rapidly self-cured, most by two weeks. In contrast another species, *Litoria ewingii*, developed severe chytridiomycosis when exposed to the same inoculum.

As inhibition by skin bacteria has been suggested as a factor in resistance to Bd, I investigated baseline cutaneous bacterial flora in native NZ frogs. Ninety-two unique bacterial isolates were identified from the ventral skin of 62 apparently healthy *L. archeyi* and *L. hochstetteri* frogs from the Coromandel and Whareorino regions in New Zealand were identified using DNA extraction and

polymerase chain reaction techniques. A New Zealand strain of Bd (KVLe08SDS1) was also isolated for the first time from a *Litoria ewingii* from the Dunedin area. This Bd strain was used against 21 bacterial isolates in an *in vitro* challenge assay to test for Bd inhibition. One bacterial isolate, a *Flavobacterium* sp., inhibited the growth of Bd. This positive result may indicate that cutaneous bacteria are part of the innate immunity of *L. archeyi* against chytridiomycosis and is the first report of its kind in *Leiopelma* spp.

In conclusion, captive leiopelmatids had high mortality rates due to inadequate husbandry. To prevent multi-factorial MBD in captive *Leiopelma* spp., dietary calcium should be increased, exposure to ultraviolet-B light increased and de-fluoridated water used as a minimum standard. Attempting to recreate natural diets and conditions will improve the chances of establishing a healthy breeding collection. Chytridiomycosis was not identified as a cause of death in any captive cases. Amphibian chytrid is geographically widespread in New Zealand and has been found in all *Litoria* spp. and *L. archeyi*. Populations of *L. archeyi* infected with Bd appear to be stable at present and as individuals self-cured when reinfected in captivity, this species appears to have some natural resistance to chytridiomycosis. In contrast, populations of non-native *Litoria* spp. have generally declined. Cutaneous bacteria of *L. archeyi* may play a role in their innate immunity. Bd has not been found in any other leiopelmatids despite widespread testing. Hence chytridiomycosis does not appear to be a current threat to *L. archeyi* or *L. hochstetteri*, although further surveys are needed to understand population impacts on *L. archeyi.* The continued use of field hygiene protocols to reduce the risk of introducing Bd (or new strains in the case of populations where it is already present) or other pathogens to threatened frog populations are recommended.

This project has exemplified the importance of integrating the baseline data obtained from healthy wild-caught frogs to aid disease investigation of captive frogs. It also demonstrates the value of both clinical disease experience and an ecological viewpoint when investigating and managing disease in wildlife.

Abbreviations

AH	adenomatous hyperplasia
Bd	*Batrachochytrium dendrobatidis*
DOC	Department of Conservation
GIS	global information system
HZQ	Hamilton Zoo quarantine
IPX	immunoperoxidase
JCU	James Cook University
MBD	metabolic bone disease
PCR	polymerase chain reaction
spp.	species (plural of sp.)
SNP	single nucleotide polymorphisms
TEM	transmission electron microscopy
UVB	ultraviolet-B light

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Chapter 1: Introduction

New Zealand has four extant native frogs and all are in the genus *Leiopelma*: Archey's frog (*Leiopelma archeyi*), Hamilton's frog (*Leiopelma hamiltoni*), Hochstetter's frog (*Leiopelma hochstetteri*) and the Maud Island frog (*Leiopelma pakeka*). They are all nocturnal, terrestrial direct-developing frogs with the exception of *L. hochstetteri*, which is semi-aquatic (Bell, 1978; Bell and Wassersug, 2003). They range in size from 25 to 47mm sout-vent length (SVL) and average 4-8g in seight, depending on species, with *L. archeyi* being the smallest and *L. pakeka* the largest (P. Bishop, unpubl. data). They all possess unique and evolutionary primitive traits: vestigial tail-wagging muscles, cartilaginous inscriptional ribs, the presence of amphicoelous vertebrae, and nine presacral vertebrae (versus the normal eight) (Bishop et al., 2008). Worldwide, all four species are in the top 100 of the Evolutionary Distinct and Globally Endangered list with *L. archeyi* listed in the top position (E.D.G.E., 2011). They also have the following IUCN threat classifications: *L. archeyi* (critically endangered), *L. hamiltoni* (endangered), *L. hochstetteri* (vulnerable) and *L. pakeka* (vulnerable) (IUCN, 2011). They are listed in New Zealand from threatened to critically endangered (Hitchmough et al., 2005).

In 2005 the New Zealand Department of Conservation (DOC) Native Frog Recovery Group (NFRG) convened at Auckland Zoo for two days to discuss two areas of growing concern. One, there appeared to be high mortality in the captive frogs, but the data was scattered and the situation was difficult to decipher. Secondly, that the amphibian chytrid fungus, which was thought to have caused a ten-fold population decrease in the Coromandel population of the critically endangered Archey's frog (Bell et al., 2004) had now been found in the only other population in the Whareorino forest. In addition, the disease status and threat of amphibian chytrid to all the other *Leiopelma* spp. populations was completely unknown. Hence, this PhD thesis was motivated by two general concerns: 1) the overwhelming lack of knowledge about diseases in *Leiopelma* spp.; and 2) a lack of organised research to provide the evidence on disease to enable the best decisions to be made to conserve *Leiopelma* spp.

This PhD thesis answers two basic questions:

1) What is the health status of the captive *Leiopelma* spp. and what diseases, if any, are limiting their survival?; and

2) Is the amphibian chytrid a threat to free-ranging *Leiopelma* spp.?

Some reports and studies on *Leiopelma* spp. have been done on the ecological factors affecting the population size such as habitat, pesticides, and predators (Baber et al., 2006; Bell, 1994; Bell and Pledger, 2010; Haigh et al., 2007; Perfect and Bell, 2005; Thurley, 1996; Tocher et al., 2006; Ziegler, 1999). However, in the face of the global amphibian decline, little investigation has been done on what diseases, including amphibian chytridiomycosis, affect *Leiopelma* spp. populations in the wild.

The first step to my thesis was a literature review collating what diseases or possible disease aetiologies had been reported in *Leiopelma* spp. and the introduced *Litoria* spp. in New Zealand up to 2008. Only eight relevant papers were located which were all observational studies in the form of case reports or case series.

Parasites

Nematodes

The first paper to report nematodes in native frogs described general field observations in *L. archeyi* and *L. hochstetteri* infected with undescribed members of the Cosmocercinae (Stephenson and Stephenson, 1957). There was no information on numbers of frogs examined or if any pathology was associated with nematode infections. Baker and Green (1988) examined three native free-living frog species: *L. archeyi, L. hamiltoni* and *L. hochstetteri*. The nematodes *Aplectana novaezelandiae* and *Cosmocerca australis* were both new species from the subfamily Cosmocercinae found in *L. hochstetteri* although the exact number of frogs examined out of the 50 collected was not recorded. Another new species, *Cosmocera archeyi* from the subfamily Cosmocercinae, was found in one *L. archeyi* out of five frogs possibly examined. There were no parasites found in the three *L. hamiltoni* examined. Again, there was no information or comments on the prescence of any pathology associated with the nematode infections (Baker and Green, 1988).

Trematodes

Dolichosaccus novazealandiae, a digenean trematode, was described as a new species in both *L. archeyi* and *L. hochstetteri* (Prudhoe, 1970). Further description of this trematode species in *L. hochstetteri* appeared in two more publications (Allison and Blair, 1987; Baker and Green, 1988). Amy Hackner, a Unitec student doing a faecal survey as a Bachelors of Applied Animal Technology project, examined 51 samples from captive *L. archeyi* at the Auckland Zoo in 2005-2006 and only found one egg from *D. novazealandiae*. She also examined 31 samples from free-living *L. archeyi* from the Whareorino area and found no endoparasites (Hackner, 2006).

Cestodes, Protozoa and Haemoparasites

None have been reported in the literature.

Chytridiomycosis

Chytridiomycosis is a fungal skin disease caused by the amphibian chytrid, *Batrachochytrium dendrobatidis* (Bd) (Berger et al., 1998). This skin infection has caused massive amphibian declines worldwide (Bosch et al., 2001; Daszak et al., 1999; La Marca et al., 2005; Lips, 1999; Skerratt et al., 2007) and was first found in New Zealand in a non-native frog species, *Litoria raniformis*, in 1999 (Waldman et al., 2001). Bd was again found in 2001, but this time in *L. archeyi* in the Coromandel peninsula region and was associated with a population decline (Bell et al., 2004). Although a few dead frogs were found infected with Bd in the area, the link was considered circumstantial as Bd was not found in the first decline in 1995 and 1080 poison was also used in the area. However, the effect of 1080 poison on leiopelmatid frogs is probably minimal (Bell et al., 2004; Perfect and Bell, 2005). As the amphibian chytrid has caused declines worldwide it appears to be a likely cause of the *L. archeyi* decline, but the evidence is still unclear.

Other diseases

Potter and Norman (2006) provide the first report of clinical problems in captive *L. archeyi*, based on frogs at Auckland Zoo. It is largely a descriptive paper discussing four main clinical syndromes seen: skin blisters, cloudy corneas, weight loss and extensor spasms. It also gives a description of various treatment regimens tried. The paper was important in raising awareness that these small frogs were undergoing significant morbidity and mortality and that little was known of the aetiologies of these diseases of captive frogs. The one problem with this paper is that it is in a journal that is not available online and not held by many libraries so access can be difficult.

Review of Chapter Content

Chapter Two addresses the first main thesis question, "What is the health status of the captive *Leiopelma* spp., and what diseases, if any, are limiting their survival?" and includes a published epidemiological analysis of mortality in captive frogs. The overall aims of this chapter were to:

1) collate the information and verify the high mortality reported;

2) identify trends of mortality by species, husbandry factors, year of death, collection site, transfer cohort, sex and cause of death; and

3) identify any causes of morbidity and mortality so that recommendations could be made to improve management.

This analysis was done in an "information vacuum" to some extent, hence looked at mortality trends rather than specific diseases which were poorly known at that time.

Chapter Three originated as part of the investigation into the captive diet and its role in metabolic bone disease. However, it expanded into a broader study to include an analysis of the

stomach contents of wild frogs killed by misadventure to assist in the formulation of an improved captive diet. The aims of this chapter were to:

1) describe the invertebrate fauna ingested by free-ranging native frogs;

2) compare the diet of free-ranging frogs to that of captive frogs; and

3) make recommendations on how to improve the captive diet, based on the assumption that the wild diet was superior.

Chapter Four then concentrated on the major disease syndrome, metabolic bone disease (MBD), which was identified as a problem at all captive institutions. The overall aims of this chapter were to:

1) calculate the prevalence of metabolic bone disease in the captive *Leiopelma archeyi* and *Leiopelma hochstetteri* populations;

2) diagnose the aetiology of the disease; and

3) make recommendations for prevention.

This investigation was an exhaustive one involving data collected from three captive facilities and collaboration with three diagnostic institutions. I described how exposure to fluoride in the water played a major role in MBD in these frogs which was previously undescribed in amphibians.

As part of the ongoing investigation on the causes of mortality of captive *Leiopelma* spp., **Chapter Five** concentrated on the occurrence of a novel nematode and protozoal nasal discharge that was associated with morbidity and mortality in a small number of captive Archey's frogs at Auckland Zoo. This chapter had several aims:

1) to assist other veterinarians who work with amphibians by describing clinical signs, laboratory investigation and specific treatment for these parasites and

2) to describe the three organisms associated with this infection.

Two of the organisms found in the frogs had not been associated with nasal infections in amphibians previously, and none had ever been described in frogs in New Zealand.

Chapter Six is the last part of the investigation into the overall health of captive *Leiopelma* spp.. It details an investigation into the "blister syndrome" which was previously described in a veterinary journal with limited access (Potter and Norman, 2006). This chapter had three aims:

1) to investigate and describe the epidemiology, gross pathology, histology, and ultra-structure of the lesions;

2) to determine the aetiology of the syndrome; and

3) to make recommendations for treatment and prevention if necessary.

The thesis then switches focus to the second main question of the project: Is the amphibian chytrid a threat to free-ranging native frogs?

Chapter Seven is a unique chapter in this thesis as it involves the three non-native frog species in New Zealand (*Litoria aurea*, *Litoria ewingii* and *Litoria raniformis*). Citizen science was used to obtain qualitative historical data about population trends of frogs. The aims of this chapter were to:

1) confirm the anecdotal rumours that frogs in New Zealand are in decline and if so, to identify the location and timing of any declines and any associated factors;

2) identify growing or stable populations of *Litoria* spp. which may assist future disease surveys or population monitoring and also to identify sources of genetic material that may serve as an Ark for declining Australian populations; and

3) identify suitable regions for translocations of *Leiopelma* spp. where *Litoria* spp. populations may be absent, which would reduce the risk of disease transmission from non-native to native species.

Chapter Eight provides a critical part of the puzzle of chytridiomycosis in New Zealand as it describes the distribution and prevalence of Bd in New Zealand spanning surveys from 1930 through to 2010. Collating this information was paramount as I was then able to identify and fill in gaps in the records in native frog populations by organizing additional sample collection and testing. Many scattered, unpublished Bd records were obtained to produce a large dataset that can be maintained separately, but can also be amalgamated into the Australian Bd database (Murray et al., 2010). This will ensure that the unpublished data is available for use in further epidemiological studies.

Chapter Nine is a crucial laboratory experiment in this thesis as it tested the susceptibility of *L. archeyi* to chytridiomycosis. These results assisted in the general understanding of how chytrid could be affecting wild population and had many management implications.

Chapter Ten identified the normal bacterial skin flora of *L. archeyi* and *L. hochstetteri* and investigated their role in innate immunity to chytridiomycosis. The aims of this chapter were to:

1) establish baseline bacterial skin flora in free-living native frogs; and

2) test some of these bacterial isolates *in vitro* against a New Zealand isolate of amphibian chytrid to identify any bacteria that could inhibit its growth.

Chapter Eleven reviews the major outcomes of the thesis in response to the two central questions of the thesis and gives both management and research recommendations.

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Chapter 2: Mortality of New Zealand native frogs in captivity

Preamble

This chapter is an epidemiological analysis of mortality in captive leiopelmatid frogs. The study was done in response to the concern of the New Zealand Department of Conservation (DOC) regarding the apparent high mortality in the captive population of *Leiopelma archeyi* and *Leiopelma hochstetteri* at Canterbury University.

The overall aims of this chapter were to:

- collate the information and verify the high mortality reported;
- identify trends of mortality by species, husbandry factors, year of death, collection site, transfer cohort, sex and cause of death; and
- identify any causes of morbidity and mortality so that recommendations could be made to improve the current situation.

For my analyses I reviewed the mortality records, husbandry records and histopathology reports from captive frogs from 2000 to 2005. In 2000 a large number of native frogs were brought to the University of Canterbury for captive breeding and disease research, and in 2005 they were transferred out of that facility due to the conditions of their permit being violated. This analysis was done in an "information vacuum" to some extent given that it did not look at mortality due to specific diseases which were largely unknown at that time. Hence, it analysed mortality only in an attempt to highlight factors that might be influencing mortality.

The main issue with this paper was the lack of consistent, quality data and the inability to obtain better data since it was a retrospective study. The available data from Canterbury University was often incomplete and not consistent for each frog. Due to the circumstances surrounding the transfer of

these frogs (a legal case was in progress), investigation of the past conditions was restricted. The mortality causes were only available on some frogs in the form of pathology reports, as not all had been necropsied. These causes were also difficult to interpret as they were mainly histological diagnoses, which can be misleading as sometimes the captive conditions or the gross post-mortems can give a better diagnosis to the primary causes of death. Our approach then was to use a simplistic descriptive approach and use statistics when possible, keeping the limitations of the data in mind.

Overall, the report did satisfy the original objectives of DOC by summarizing the captive conditions of the frogs, analysing the mortality data and pointing out what was known that may have attributed to the mortality of these frogs so improvements could be made. The paper went through a lengthy scientific and editorial review within DOC's publishing house. This chapter is the original governmental report as published for the DOC Research and Development Series: Shaw S.D., Holzapfel A. (2008). Mortality of New Zealand native frogs in captivity. DOC Research and Development Series 295.

Available at http://www.doc.govt.nz/upload/documents/science-and-technical/drds295.pdf.

My contribution: 90% (detailed in co-author publication release form at the end of this chapter).

Mortality of New Zealand native frogs in captivity

Stephanie Shaw and Avi Holzapfel

DOC RESEARCH & DEVELOPMENT SERIES 295

Published by
Science & Technical Publishing
Department of Conservation
PO Box 10420, The Terrace
Wellington 6143, New Zealand

DOC Research & Development Series is a published record of scientific research carried out, or advice given, by Department of Conservation staff or external contractors funded by DOC. It comprises reports and short communications that are peer-reviewed.
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ISSN 1176–8886 (hardcopy)
ISSN 1177–9306 (web PDF)
ISBN 978–0–478–14455–0 (hardcopy)
ISBN 978–0–478–14456–7 (web PDF)

This is a client report commissioned by Waikato Conservancy and funded from the Science Advice Fund. It was prepared for publication by Science & Technical Publishing; editing and layout by Amanda Todd. Publication was approved by the General Manager, Research & Development Group, Department of Conservation, Wellington, New Zealand.

In the interest of forest conservation, we support paperless electronic publishing. When printing, recycled paper is used wherever possible.

CONTENTS

Mortality of New Zealand native frogs in captivity

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ABSTRACT

Three species of New Zealand native frogs (*Leiopelma archeyi*, *L. hochstetteri* and *L. pakeka*) have been held in captivity in various institutions since 2000 as part of a programme to maintain and breed these threatened species. Following the death of a large number of these captive frogs, the Department of Conservation Native Frog Recovery Group decided that an investigation was needed to determine the cause. In this mortality study, we obtained data from captive populations to analyse mortality rates and causes of death for 252 wild-caught *Leiopelma* spp. These were held in captivity at the University of Canterbury and later transferred to other institutions between 2000 and 2006. *Leiopelma archeyi* and *L. hochstetteri* had similar overall average mortality but different yearly mortality patterns, whereas *L. pakeka* had much lower overall mortality. The major cause of death for *L. archeyi* and *L. hochstetteri* was bacterial infection, which was thought to be induced by a combination of husbandry factors, but mainly from oversterilising the substrate. Consequently, Auckland Zoo instigated a change in management, whereby soil was only heated to a temperature and for a length of time that was just sufficient to kill amphibian chytrid. New disease syndromes (skin blisters and muscle deterioration (rhabdomyolysis)) were also detected. Knowledge of disease is an important component of captive husbandry, so that healthy breeding populations can be maintained and we can gain an insight into diseases that may be affecting free-living populations. It is recommended that the staff at each institution undertake a review of all captive mortalities and report back to the Native Frog Recovery Group on an annual basis, so that any husbandry or disease issues that have arisen can be identified quickly.

Keywords: *Leiopelma*, amphibian disease, captive management, New Zealand, frogs, amphibian chytrid, rhabdomyolysis, septicaemia, blisters

Shaw, S.; Holzapfel, A. 2008: Mortality of New Zealand native frogs in captivity. *DOC Research & Development Series 295*. Department of Conservation, Wellington. 30 p.

1. Introduction

The New Zealand frog fauna currently comprises four species of the genus *Leiopelma*: *L. archeyi* (Archey's frog), *L. hamiltoni* (Stephens Island frog), *L. hochstetteri* (Hochstetter's frog) and *L. pakeka* (Maud Island frog) (Bishop & Germano 2006). All four species are considered threatened (Hitchmough et al. 2007) and permits are required from the Animal Ethics Committee, Department of Conservation (DOC) to manipulate these animals for research purposes. As part of DOC's native frog recovery programme, captive populations of all species except *L. hamiltoni* have been established in a number of localities, either to aid research or for breeding.

During 1996–2001, a major population decline of *L. archeyi* occurred on the Coromandel Peninsula, which was possibly associated with *Batrachochytrium dendrobatidis* (*Bd*)—the amphibian chytrid (Bell et al. 2004). The only other population of *L. archeyi* was located in Whareorino, where no decline or amphibian chytrid had been detected. Therefore, since *Bd* was thought to be the cause of the decline, a new captive population of *L. archeyi* from Whareorino was also established at the University of Canterbury (CU) in response to this perceived threat of disease.

Up until 2004, the majority of native frog captive populations were held at CU. However, in late September 2004, a decision was made to move all species to separate institutions; this was achieved over the following 2 years. All living *L. archeyi* from CU were moved to Auckland Zoo (AZ); these consisted of progenies from both Coromandel Peninsula and Whareorino populations. All living *L. hochstetteri* were moved to Hamilton Zoo (HZ). Twelve living *L. pakeka* were moved to the University of Otago (OU) for further study and 30 were transferred to Karori Wildlife Sanctuary, Wellington, for release.

It was known that many frogs had died at CU, but no one had yet looked at the data to identify causes. In addition, a large number of *L. archeyi* died in the first year of arrival at AZ. Following these deaths in captivity, the DOC Native Frog Recovery Group decided that an investigation was needed to determine their cause. In this mortality study, we obtained data from each institution holding native frogs in captivity to examine the relationship between mortality rate and species, husbandry technique, duration in captivity, collection site and sex. We also investigated cause of death.

2. Methods

This study used information from records between 26 November 2000 and 27 November 2006. Therefore, it excludes data regarding the Whareorino population of *L. archeyi* that was caught from the wild in late 2006, individuals from which are now held at both Auckland Zoo and the University of Otago.

2.1 ACQUISITIONS, TRANSFERS AND DEATHS

All raw data on acquisitions, transfers and deaths of individual frogs were compiled and verified by DOC staff, based on collection labels, field notes and correspondence. Any data entries that were uncertain or unverifiable (i.e. not labelled at all, collection date not clear, date of death unclear, identification uncertain) were excluded.

2.2 HUSBANDRY

Although the DOC Native Frog Recovery Group has produced a husbandry manual for keeping native frogs in captivity (Webster 2002), the exact method of husbandry varies between captive institutions and with species. Therefore, each of the institutions that held frogs was asked to complete the same questionnaire (Appendix 1). One institution chose not to participate. Any follow-up clarifications that were required were obtained by email or phone. The University of Canterbury was not given a questionnaire to be filled out initially, as the principal investigator was no longer available; however, each question was later asked by email to the primary caretaker of the frogs, as it was decided that the comparison data would be useful. Based on the responses, key parameters of each institution's frog management methods were categorised and summarised.

2.3 MORTALITY RATE

To identify any patterns in frog mortality, the raw data were analysed by species, year, number of days in captivity, collection group (frogs from generally the same time and place) and transfer cohort. The two populations of *L. archeyi* were analysed separately. Age was not included as it was unknown at the time of capture. Snout-vent length was also not used, as few data were available for that parameter.

2.3.1 Mortality rate by year

Mortality rate measures the rapidity with which new deaths occur over time. However, since often the exact time of death was unknown, an estimation was made that used a denominator that represented the average number of frogs at risk. This was calculated for each year as:

$$M = \frac{D}{(n_1 + n_2)/2}$$

where M = mortality rate, D = total number of deaths for each year, n_1 = number of frogs at the start of each year, and n_2 = number of frogs at the end of each year.

This calculation was made for each species and was expressed as a percentage. This was used to indicate whether mortality events were associated with a particular period of time (Thrushfield 2007).

2.3.2 Mortality rate by days in captivity

To investigate the relationship between the length of time an individual had been in captivity and mortality rate, the number of days from collection to death was counted for each individual of a species and categorised. The categories were up to 90 days in captivity, 180 days in captivity, and then every 180 days through to 1980 days in captivity. The number of dead individuals divided by the number of live individuals at the beginning of that time period gave a cumulative mortality rate for each category.

2.3.3 Mortality rate by collection group (CG)

To determine whether the capture circumstances influenced mortality, each significant collection (five or more individuals) of frogs from approximately the same time and place was identified by a collection group (CG). The mortality rate for each CG was calculated by dividing the number of individuals that died for each CG over the total number of frogs in that CG. Nearly all collection groups of *L. archeyi* and *L. hochstetteri* were from Coromandel, and the majority were from a single area (Tapu); the only exception was a collection from Whareorino in 2002. Although *L. pakeka* had two significant cohorts, these were not analysed, as it had already been determined that their mortality was very low so further analyses would not be worthwhile.

2.3.4 Mortality rate by transfer cohort

Leiopelma archeyi were transferred from CU to AZ in four cohorts, with each transfer differing to some degree in regards to substrate and handling. Three of these comprised only individuals from one population while the last cohort was a mix of individuals from Coromandel and Whareorino. The quarantine substrate (3 months) was either paper towels or soil, while the post-quarantine substrate was soil in all cases. Some CU frogs in the initial transfer cohort were found to have skin blisters of unknown aetiology prior to transfer. Therefore, as a precaution, all blistered frogs were housed separately from non-blistered frogs, and all following cohort transfers contained either only frogs with blisters or only those without.

To examine whether transfer technique affected mortality, the following equation was used (Thrushfield 2007):

$$M = \frac{D}{(n \times t)/12}$$

where M = mortality rate, D = total number that died from each cohort, n = the total number in that cohort, and t = the number of months that cohort was present.

Since *L. pakeka* and *L. hochstetteri* were transferred in a single cohort, they were not analysed in this way.

2.3.5 Mortality rate by sex

The sex of individual frogs was determined either by post-mortem at Massey University (MU) (for all deaths that occurred at AZ), or by CU staff, who used a combination of methods, including observing eggs in females, ultrasound and/or post-mortem for frogs that died there. Since different protocols were used, MU and CU data were analysed separately as well as combined (to increase the sample size). The mortality rate for each sex and species was calculated as the number of animals of known sex that died divided by the total number of known sex individuals.

2.4 PATHOLOGY

Pathology reports for the period covered by this study had been prepared by Dr Richard Norman at Massey University. A summary of all reports from native frogs that died in captivity was prepared. We attempted to group causes of death into general categories, e.g. a 'bacterial' category, which consisted of bacterial infections of the skin, gastrointestinal tract and coelom. All the dead *Leiopelma* from CU not yet necropsied are stored in preservative at the DOC Waikato Conservancy Office awaiting post-mortems.

3. Results

3.1 ACQUISITIONS, TRANSFERS AND DEATHS

In total, 252 individual frogs (106 *L. archeyi*, 100 *L. hochstetteri* and 46 *L. pakeka*) were brought into captivity at CU between 2000 and 2004 (Appendix 2). These were mainly obtained from the wild; the exception was four *L. archeyi* and seven *L. hochstetteri*, which first went to VU and were then transferred to CU.

In 2005 and 2006, 154 frogs (67 L. *archeyi,* 45 *L. hochstetteri* and 42 *L. pakeka*) were transferred live from CU to another institution.

In total, 113 frogs (54 *L. archeyi*, 55 *L. hochstetteri* and 4 *L. pakeka*) died while in captivity.

3.2 HUSBANDRY

Key husbandry parameters varied between institutions, particularly with respect to group housing (Table 1, Appendix 3). All of the institutions kept individual animals in separate, small plastic containers on paper towels. However, the group housing varied from indoor on paper towels to outdoor on natural substrate. Two institutions (UC and HZ) sourced all the natural substrate components locally rather than from the original habitat of the species, whereas AZ sourced the soil and leaf litter from Coromandel. The method used to sterilise the leaf litter and soil also varied.

3.3 MORTALITY RATE

The frogs included in this study had been captured and brought into captivity mainly for research and captive propagation. For some, the specific research purpose was known, e.g. *L. archeyi* (Coromandel) were intended for amphibian chytrid studies. However, according to databooks and notes at CU, no invasive research ever took place that could be considered to have affected their mortality. The only known exception to this was four *L. archeyi* (Coromandel) and seven *L. hochstetteri* that were collected in 2002 and initially went to VU for manipulative chytridiomycosis research. These were part of a larger collection of frogs (ten of each of the two species), the rest of which died at VU. Three of the four *L. archeyi* and four of the seven *L. hochstetteri* that were transferred from VU subsequently died at CU, and it is possible that they arrived at CU in a weakened state. Nine of the 46 *L. pakeka* and two *L. archeyi* collected were brought into captivity over some health concerns (dermatitis, eye problems, blisters, bleeding or head injury). All of these individuals except the one frog with a head injury survived for the duration of this study.

TABLE 1. HUSBANDRY BASICS AT EACH CAPTIVE FACILITY. FOR ADDITIONAL INFORMATION, SEE APPENDIX 3.

PARAMETER	INSTITUTION			
	UNIVERSITY OF CANTERBURY*	AUCKLAND ZOO	HAMILTON ZOO	UNIVERSITY OF OTAGO†
Temperature				
Group	Controlled; 11–15°C	Controlled; 12–16°C	Ambient	Controlled; 12–16°C
Individual	Controlled; 11–15°C	Controlled; 12–16°C	Air conditioning; 12–15°C	Controlled; 12–16°C
Humidity				
Group	Unknown	Controlled; 100%	Ambient	Controlled; >85%
Individual	Unknown	Controlled; 100%	Ambient	Controlled; >85%
Watering				
Group	Automatic—ceramic filtered	Manual—reverse osmosis	Automatic—carbon filtered	Automatic—filtered 2 µm
Individual	Manual moistening—ceramic filtered	Manual—reverse osmosis	Unknown	Automatic—filtered 2 µm
Lighting				
Group	Fluorescent light; 12 h cycle	Incandescent bulb; 12 h cycle	Ambient	Fluorescent; 11 h ramped on
Individual	Fluorescent light; 12 h cycle	2 bulbs during day, 1 filtered at night	Ambient	Fluorescent; 11 h ramped on
Handling				
Group	Weekly	2–6 times/month	Twice a month	Monthly
Individual	Weekly	Weekly	Weekly	Monthly
Substrate				
Group				
Source	Local origin	Tapu, Coromandel	Local origin	Maud Island
Preparation	Autoclaved; dried at 140°C for 72 h	Baked at 200°C, then sun-dried for 90 days	Dried at >20°C for 14 days	150°C for 3 h, acclimatised for 30 days
Individual	Paper towels	Paper towels	Paper towels	Paper towels
Feeding				
Group	Weekly	Twice a week	Three times a week	Weekly
Individual	Weekly	Twice a week	Three times a week	Weekly

* University of Canterbury had seven frog areas and conditions could vary; information is based on main holding areas.

† University of Otago had group tanks prepared but had not used them at the time of this paper.

3.3.1 Mortality rate by year

Mortality rates by year and species are shown in Fig. 1.

Leiopelma archeyi (Whareorino) had an average mortality rate of 14.5% across all years. Mortality rate was lowest in the first (2002 = 12.2%) and third (2004 = 7.8%) years of significant holdings (i.e. year in which there was more than one frog in the captive population). Mortality rate was higher in the second and fourth years (2003 = 20.5%; 2005 = 24.2%), and was highest of all in the last year (2006 = 36.7%), when frogs were transferred to AK.

Leiopelma archeyi (Coromandel) had an average mortality rate of 10.3% across all years, which was about half that of *L. archeyi* (Whareorino). Mortality rate was highest in the third and fourth years of holdings (2002 = 17.9%; 2003 = 27.8%), which accounted for most of the overall deaths. Since the fifth year (2004), the

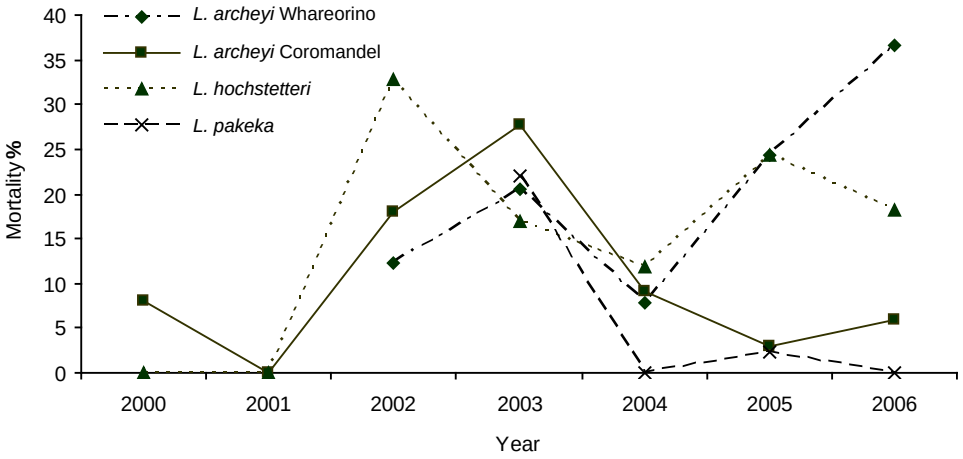

Figure 1. Mortality rate of *Leiopelma* spp. by year.

mortality rate has been at or below the average rate; this includes during and following the transfer to AK.

Leoiopelma hochstetteri had an average mortality rate of 14.9% across all years. No deaths occurred in *L. hochstetteri* in the first 2 years of holdings (2000 and 2001); since then, the mortality rate has fluctuated between 11.8% and 24.4%. No deaths occurred at HZ during the initial 6 months following transfer.

Leiopelma pakeka had an average mortality rate of 3.5% across all years, which was the lowest of all the species. The mortality rate was highest in the first year of significant holdings (2003); since then, the mortality rate has been very low (2.4 % in the third year (2005) and 0.0% in other years), including during and following the transfer to OU.

3.3.2 Mortality rate by days in captivity

Mortality rate by the total number of days in captivity for each species is shown in Fig. 2. Since cumulative data are presented, increases in the slope of the lines indicate where deaths mainly occurred.

For *L. pakeka*, all deaths occurred within the first 1.5 years (540 days) of captivity (8.7%). Rates of mortality for the remaining three taxa were higher than *L. pakeka* but were broadly similar to each other for the initial 900 days in captivity; following this, each species had different mortality rates.

Leiopelma archeyi (Whareorino) had a low mortality rate (1.9%) in the first 90 days of captivity. Following this, there were fairly uniform increases in overall mortality from day 90 to day 1620 (between 5.7% and 11.6%), except for the lower increases (1.9%) between 540 and 900 days in captivity. The mortality rate was very low for those frogs that had been in captivity longest, i.e. between 1620 and 1980 days, with between 0.0% and 1.2% increases in overall mortality rate.

Leiopelma archeyi (Coromandel) had low increases in mortality rate (2.1% and 2.2%) up to 180 days in captivity. From 180 to 900 days, increases were between 6.3% and 12.7%, except for the low increases (2.2%) between 360 and 540 days. After 900 days, increases in mortality rate were consistently low (between 0.0% and 2.2%).

Leiopelma hochstetteri had continual increases in overall mortality rate through time, varying from 2.1% to 7.6%.

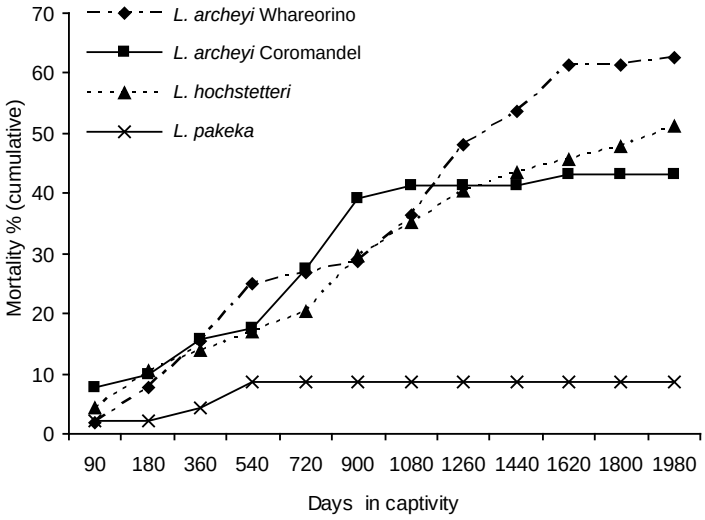

Figure 2. Mortality rate of *Leiopelma* spp. by total number of days in captivity.

3.3.3 Mortality rate by collection group (CG)

Mortality rates by collection groups are shown in Table 2.

All three *L. archeyi* (Coromandel) collection groups had similar mortality rates (between 35.7% and 40.0%). However, their average mortality rate (38.6%) was about half that of the Whareorino collection group of the same species (65.3%).

The range of mortality rates amongst the *L. hochstetteri* collection groups was similar to that of both populations of *L. archeyi* combined (ranging from 28.6% to 65.2%), and the overall average (47.2%) was about halfway between the two populations of *L. archeyi*. Mortality rate was highest for the oldest two collections and lowest for the second-youngest collection.

TABLE 2. MORTALITY RATE OF *Leiopelma archeyi* AND *L. hochstetteri* BY COLLECTION GROUP (CG).

CG	NO. INDIVIDUALS IN CG	YEAR OF DEATH							TOTAL DEATHS	% MORTALITY
		2000	2001	2002	2003	2004	2005	2006		
L. archeyi										
Coromandel 2000	30	1	0	5	5	1	0	0	12	40.0%
Coromandel 2002	14	0	0	0	3	1	0	1	5	35.7%
Coromandel 2004	5	0	0	0	0	0	1	1	2	40.0%
Total Coromandel	49	1	0	5	8	2	1	2	19	38.6% (average)
Whareorino 2002	49	0	0	3	9	3	8	9	32	65.3% (average)
L. hochstetteri										
Coromandel 2000	23	0	0	4	5	1	2	3	15	65.2%
Coromandel 2002	28	0	0	4	2	3	7	1	17	60.7%
Coromandel 2003 A	12	0	0	0	3	0	1	1	5	41.7%
Coromandel 2003 C	14	0	0	0	0	0	1	3	4	28.6%
Coromandel 2004	10	0	0	0	0	3	0	1	4	40.0%
Total Coromandel	87	0	0	8	10	7	11	9	45	47.2% (average)

3.3.4 Mortality rate by transfer cohort

Mortality of the third cohort of *L. archeyi* from Whareorino that was transferred was about twice as high as the two other cohorts from the same population (64.0% v. 54.5% v. 150.0%, for cohorts 1–3 respectively). The later cohort from Coromandel also had a higher mortality rate (Table 3). All three Whareorino cohorts had higher mortality rates than the Coromandel cohort.

Individuals from both the Whareorino and Coromandel cohorts that had blisters had higher mortality rates than those with no blisters. The one Whareorino cohort that had a mixture of blistered and non-blistered individuals had a similar mortality rate as the Whareorino cohort with no blisters. The quarantine substrate did not appear to have influenced mortality.

TABLE 3. MORTALITY RATE BY TRANSFER COHORT OF *Leiopelma archeyi* DURING TRANSFER FROM CANTERBURY UNIVERSITY TO AUCKLAND ZOO.

W= Whareorino; C= Coromandel; P= papertowels; S = natural substrate; N= no; Y= yes.

	TRANSFER COHORT				
	1	2	3	4a	4b
Population	W	C	W	W	C
Date of arrival	15 Mar 05	10 June 05	4 July 05	9 Nov 05	9 Nov 05
Quarantine substrate	P	P	S	P	8P/8S
Post-quarantine substrate	S	S	S	S	S
Blistered	Some	N	N	Y	Y
Total in transfer cohort	15	17	11	8	16
Total that died	8	1	3	2	1
% mortality rate	64.0%	10.1%	54.5%	150.0%	37.5%

3.3.5 Mortality rate by sex

Females consistently outnumbered males in the number of deaths for both *L. archeyi* and *L. hochstetteri* (Table 4).

For *L. archeyi*, 80% of individuals that died were female, regardless of whether sex was determined by a single method/institution or a combination of methods/ institutions.

For *L. hochstetteri*, 84% of individuals that died were female.

TABLE 4. MORTALITY RATE BY SEX OF *Leiopelma archeyi* AND *L. hochstetteri*.
AZ = Auckland Zoo; CU = University of Canterbury; MU = Massey University. Data exclude froglets.

	FEMALE	MALE	UNKNOWN	TOTAL OF KNOWN SEX	TOTAL NO. INDIVIDUALS
***L. archeyi* from AZ (sexed by MU)**					
Number dead	11	3	2	14	16
Dead/dead of known sex	78.6%	21.4%			
***L. archeyi* from CU (sexed by MU and/or CU)**					
Number dead	8	2	31	10	41
Dead/dead of known sex	80.0%	20.0%			
***L. hochstetteri* from CU (sexed by MU and/or CU)**					
Number dead	21	4	27	25	52
Dead/dead of known sex	84.0%	16.0%			

3.4 PATHOLOGY

Since pathology reports were only available from early 2005 and only for a limited number of frogs that died at CU, it is probably more useful to examine trends in deaths in post-Canterbury holdings. About a third of all deaths (34.8%, $n = 16$) were attributed to bacterial causes (Table 5). The remainder of deaths were fairly evenly attributed to the other categories of causes, with 1–4 frogs (2.2%–8.7%) in each category. About a fifth (21.0%, $n = 10$) of all deaths were of unknown cause.

TABLE 5. PATHOLOGY SUMMARY FOR *Leiopelma* spp.
CU= University of Canterbury; AZ= Auckland Zoo; HZ= Hamilton Zoo; Coro = Coromandel; Whare = Whareorino.

CAUSE OF DEATH	*L .archeyi*		*L. hochstetteri*	*L. archeyi*		*L. hochstetteri*	TOTAL	%
	CORO CU	WHARE CU	CU	CORO AZ	WHARE AZ	HZ		
Bacterial (skin/gastrointestinal/coelom)	1	2	7		6		16	34.0%
Mycobacterial					1		1	2.1%
Fungal skin		1	3				4	8.5%
Kidney			1		1		2	4.3%
Trauma		1	1	1			3	6.4%
Foreign body			1	1			2	4.3%
Reproductive			1				1	2.1%
Rhabdomyolysis					3	1	4	8.5%
Eustachian tube impaction			1				1	2.1%
Ophthalmic						1	1	2.1%
Poor nutrition/weight loss			2				2	4.3%
Unknown		1	6		3		10	21.3%
Total dead with pathology reports	1	5	23	2	14	2	47	

4. Discussion

Leiopelma pakeka had the lowest overall mortality of the three species, both in terms of mortality rate by year and days in captivity. None of the data examined could explain why this species had a greater ability to withstand captures, transfers and captivity than the other *Leiopelma* species investigated. However, an earlier collection of 11 *L. pakeka* in 2000 that were exclusively housed at OU (and therefore were not included in this report) showed a much higher mortality than in this study (9 out of 11 frogs died). These deaths occurred over a brief period and were attributed to problems in husbandry (enclosure humidity, substrate and food amounts) by university staff. These factors were corrected and the remaining two frogs were still alive at the end of the period covered by this study. This indicates that husbandry conditions will influence mortality in *L. pakeka*, although perhaps not to the same degree (or for the same specific conditions) as for the other species.

The annual mortality rate of *L. hochstetteri* at CU was relatively low but steady each year. Since the population was aging each year and their start age was unknown, this decline could have been simply an aging pattern, as would be expected if all individuals in the population were of different ages. However, results indicate that husbandry conditions also played some role here. At CU, *L. hochstetteri* were kept in controlled indoor conditions. In contrast, at HZ, where they were kept in outdoor enclosures in quite uncontrolled conditions, the mortality declined in the initial 6 months to lower levels than at CU. Therefore, it seems likely that the steady decline at CU was due to husbandry factors.

The overall average mortality rate for *L. archeyi* was similar to that of *L. hochstetteri*, but annual patterns of increases and decreases differed between the species. The difference in mortality between the two populations of *L. archeyi* is also interesting, with the mortality rate of the Whareorino population being higher than that of the Coromandel population. This difference arose because although the Coromandel population consistently had higher mortality while at CU, following transfer to AZ their mortality decreased. In the Whareorino population, the reverse occurred. There are several possible reasons why *L. archeyi* Whareorino population had higher mortality than its Coromandel counterpart.

First, it is possible that the resilience of frogs differed between the two populations. For example, the single collection of Whareorino individuals may have been from a weaker population so they did not cope as well as the Coromandel individuals with the stress of being transferred and/or having a change in husbandry. Alternatively, the Coromandel individuals that were transferred may have already survived greater mortality events than the Whareorino population, both in the wild and at CU, so that the stronger individuals remained, which were better able to cope with the stress of transfer. However, the evidence does not support this, as during the first year in captivity at CU the Whareorino population had a lower mortality rate than the Coromandel population.

The second possibility is that the origin of natural substrate used in group housing had an effect on mortality. Institutions differed in which portions of the

natural substrate came from the frogs' original habitat. At AZ, the soil and leaf litter portion of the natural substrate was from Coromandel. At CU, the leaf litter portion was local, favouring neither population of *L. archeyi*. It is possible that the use of a substrate from an origin other than their original habitat could have caused an imbalance of minerals, bacteria or another unknown factor contributing to mortality. However, at times, L. *archeyi* Coromandel had higher mortality rates than Whareorino individuals at CU when neither was on native substrate. In addition, if the origin of leaf litter/soil was a major factor, *L. hochstetteri* would have been expected to have fared poorly at Hamilton Zoo, which they did not. Therefore, although there seems to be some merit in this argument of soil origin, it is likely to be a minor contributing factor rather than a primary one.

A third possibility is that susceptibility to disease had an effect on mortality. Bacterial causes (dermatitis, septicaemia and infections in the coelom) were the main single confirmed cause of death in *L. archeyi*. Primary bacterial disease is unusual in amphibians and outbreaks are often associated with a variety of situations that could result in immunosuppression, alteration of non-specific host defences, or exposure to overwhelming bacterial numbers (Pessier 2002). Even when the known primary pathogenic bacterium *Aeromonas hydrophila* is isolated, it may not be diagnostic because this and other bacteria are frequent inhabitants of frogs' environments (Pessier 2002). Bacterial septicaemia may arise as a result of the complex interaction of multiple taxa of bacteria, or the overwhelming presence of a single species (Taylor et al. 2001). In a few cases, foreign bodies of plant material had caused trauma to the skin and started an infection that eventually overwhelmed the system. Although the epidermis provides some protection from abrasive substances, it is easily damaged if the frog is handled inappropriately or is in contact with rough substances. The resulting damage from even an apparently minor injury can have serious consequences, as there is no longer an effective barrier against opportunistic micro-organisms (Helmer & Whiteside 2005). Environmental stressors may also change amphibians' bacterial skin flora (Harris et al. 2006).

The captive environment may also have had high numbers and/or new bacteria to which frogs were naïve. This exposure combined with the stress of captivity may have resulted in bacterial infections. At the different institutions, there was great variation in the temperature to which soil and leaf litter were heated in an attempt to kill any *Bd* that may have been present. Over-heating soil kills off healthy invertebrates, bacteria and fungi, leaving the soil sterile and vulnerable to colonisation of bacteria that are opportunistic invaders. Both AZ and CU used very high temperatures to sterilise the soil and subsequently kept the soil in indoor enclosures where there was little to no possibility of insects being introduced. In contrast, HZ used lower temperatures, which may have been too low to kill amphibian chytrid (Johnson et al. 2003), and the outdoor enclosure favoured easy re-colonisation by insects, etc. A comparison of bacteria present on *L. archeyi* in the wild and on the Coromandel and Whareorino populations in captivity revealed that both the captive Whareorino and Coromandel frogs had a bacterial flora on their skin that was substantially different from the flora found on free-living Coromandel and Whareorino frogs, and bacteria that have been previously implicated in contributing to disease were only found in the captive populations (Potter & Norman 2006). Therefore, it is possible that some frogs on substrate at AZ were exposed to types and numbers of bacteria that caused

disease when combined with stress or some other unknown factor. In response to these deaths, a change was made to the husbandry of the Whareorino frogs, whereby they were all placed on paper towels, in a hope that this would reduce the number of bacteria to which they were exposed. Since then, there have been no more deaths of *L. archeyi* to date.

This would lead us to conclude that the major contributing factor to the death of *L. archeyi* Whareorino was that of husbandry. Hygiene and handling protocols have always been in place to minimise the frogs' exposure to new bacteria. However, based on these findings, it is recommended that any substrate component that is sterilised for the purposes of killing chytrid is only heated for 4 hours at 37°C to preserve healthy soil that has a balanced bacterial flora.

Another disease finding in *L. archeyi* was rhabdomyolysis. This is where an acute stress or an inherited enzyme deficiency causes muscle necrosis, the degradation products of which lead to renal tubular damage and death. The preceding clinical signs of this were noted in the history of extensor spasms. As far as we are aware, this is the first report of this syndrome in an amphibian and thus it requires further investigation.

Finally, the blistering syndrome was present in many *L. archeyi* when they arrived at AZ. The third Whareorino transfer cohort and the second Coromandel transfer cohort of *L. archeyi* to AZ, which were all blistered frogs, had higher mortality rates than pure non-blistered cohorts. This was only a total of three blistered frogs, however. Blisters have been seen in both wild and captive *Leiopelma* (A. Smale, DOC, pers. comm. 2007). Blisters are a syndrome of unknown etiology that is currently under investigation. So far, this investigation has shown that they are not infectious but are believed to be caused by an immune disorder (R. Speare, James Cook University, pers. comm. 2007).

According to both the MU pathology reports and the CU notes, there was a definite female sex bias in mortality rates for *L. archeyi* and *L. hochstetteri*, notwithstanding a large number of animals (mainly those from CU) where the sex was undetermined. Similar sex-biased mortality was found in the California red-legged frog (*Rana rana*) during a decline due to chytridiomycosis (Muths et al. 2003). The hypothesis of sex-biased mortality could easily be tested for *Leiopelma* if the surviving frogs or even source populations could be reliably sexed to confirm whether there was already a sex bias in the source or collected population. Currently, snout-vent length is used to determine sex in the field (Bell 1994; Tocher et al. 2006). However, this is not very accurate, so researchers are now trying to develop a method to assign gender of individual *L. archeyi*.

5. Conclusions

Most deaths of captive frogs appear to have been caused by husbandry factors. It appears that requirements differ between species and even populations of the same species. Although the specific causes of death in each species have not been clearly identified, several possible explanations have been proposed. At the time of writing, all three species had stable mortality rates, indicating either that populations have undergone their main mortality events or that causes of death have been removed through changes in husbandry. The apparent higher mortality in female frogs in both *L. archeyi* and *L. hochstetteri* requires further scrutiny. The recent disease findings of blistering and rhabdomyolysis in *L. archeyi* also need further investigation.

6. Acknowledgements

We would like to thank Richard Jakob-Hoff, John Potter, Andrew Nelson and Peter West of Auckland Zoo, and Amanda Haigh, Waikato Conservancy, DOC. We would also like to thank Lee Skerratt and Rick Speare of James Cook University for manuscript review and statistical advice. This study was funded by the DOC Science Advice Fund, Waikato Conservancy.

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Appendix 1

Leiopelma HUSBANDRY QUESTIONNAIRE

1) Enclosure temperature day/night/seasonal and how it's monitored
2) Type of light and timing
3) Humidity and how monitored
4) Number of animals/enclosure
5) Define what the substrates you use are exactly and where you got them
6) Did the substrates get any sort of pre treatment?
7) How often are these substrates changed?
8) Describe what enclosure is made of that they have contact with, i.e. plastic container/dirt/wire mesh, etc.
9) What are your hygiene protocols? Describe brand gloves, brand disinfectants/ strength, etc.
10) Feeding regime
11) Type of water used and how delivered
12) Anything else you think pertinent?

Appendix 2

ACQUISITIONS AND TRANSFERS OF *Leiopelma* spp.

CU = University of Canterbury; AZ = Auckland Zoo; OU = University of Otago; HZ = Hamilton Zoo; VU= Victoria University of Wellington.

YEAR	INSTITUTION	SPECIES	NO. FROGS ALIVE (AT 1 JAN)	NO. FROGS ACQUIRED	SOURCE OF ACQUIRED FROGS	NO. FROGS THAT DIED (1 JAN – 31 DEC)	NO. FROGS TRANSFERRED OUT	SITE TRANSFERRED TO	DATE TRANSFERRED OUT	NO. FROGS AT END OF YEAR
2000	CU	*L .archeyi* (Whareorino)	0	1	Wild	0	0	–	–	1
		L. archeyi (Coromandel)	0	26	Wild	1	0	–	–	25
		L. hochstetteri	0	23	Wild	0	0	–	–	23
		L. pakeka	0	0	–	–	–	–	–	0
2001	CU	*L .archeyi* (Whareorino)	1	0	–	0	0	–	–	1
		L. archeyi (Coromandel)	25	2	Wild	0	0	–	–	27
		L. hochstetteri	23	5	Wild	0	0	–	–	28
		L. pakeka	0	1	Wild	0	0	–	–	1
2002	CU	*L. archeyi* (Whareorino)	1	50	Wild	3	0	–	–	48
		L. archeyi (Coromandel)	27	15+4	Wild/VU	6	0	–	–	40
		L. hochstetteri	28	29+7	Wild/VU	13	0	–	–	51
		L. pakeka	1	0	–	1	0	–	–	0
2003	CU	*L .archeyi* (Whareorino)	48	1	Wild	9	0	–	–	40
		L. archeyi (Coromandel)	40	2	Wild	10	0	–	–	32
		L. hochstetteri	51	26	Wild	10	0	–	–	67
		L. pakeka	0	20	Wild	2	0	–	–	18
2004	CU	*L. archeyi* (Whareorino)	40	0	–	3	0	–	–	37
		L. archeyi (Coromandel)	32	5	Wild	3	0	–	–	34
		L. hochstetteri	67	10	Wild	8	0	–	–	69
		L. pakeka	18	25	Wild	0	0	–	–	43
2005	CU	*L. archeyi* (Whareorino)	37	0	–	4	15	AZ	15 Mar 2005	0
							10	AZ	4 Jul 2005	0
							8	AZ	9 Nov 2005	0
		L. archeyi (Coromandel)	34	0	–	1	17	AZ	10 June 2005	0
							16	AZ	9 Nov 2005	0
		L. hochstetteri	69	0	–	15	0			54
		L. pakeka	43	0	–	1	42	OU	28 Oct 2005	0
	AZ	*L. archeyi* (Whareorino)	0	33	CU	4	0			29
		L. archeyi (Coromandel)	0	33	CU	0	0	–		33
	OU	*L. pakeka*	0	42	CU	0	0	–	–	42
2006	CU	*L. hochstetteri*	54	0	–	9	45	HZ	24 May 2006	0
	AZ	*L. archeyi* (Whareorino)	29	0	–	9	0	–	–	20
		L. archeyi (Coromandel)	33	0	–	2	0	–	–	31
	HZ	*L. hochstetteri*	0	45	CU	2	0	–	–	43
	OU	*L. pakeka*	42	0	–	0	0	–	–	42

Appendix 3

HUSBANDRY DETAILS BY INSTITUTION

Although the Department of Conservation (DOC) Native Frog Recovery Group oversees all native frog holdings, the exact methods of husbandry used for each species varies by institution. The husbandry techniques of each institution for the period of this analysis are summarised below.

A3.1 University of Canterbury

Species

This was the original holding institution for all three native frog species (*Leiopelma archeyi*, *L. hochstetteri* and *L. pakeka*). The frogs were kept in many different rooms. The main areas for group and individual housing are described below.

Group housing

Breeding groups were housed in large glass tanks with perplex and mesh lids. The frogs were not in contact with the perplex or mesh as the containers were very high. The substrate in the tanks was mainly peat and leaf litter. The peat was a commercially dried product that came in a block and was rehydrated in water, then autoclaved. The leaf litter was collected on the University of Canterbury campus (Christchurch) and was then autoclaved and dried at 140°C for 72 h. The substrate was changed as required, which was usually every 6 months.

Individual housing

All *L. hochstetteri* and *L. pakeka*, and some *L. archeyi* were kept in individual housing. Individuals that were found together in the wild were kept together in an individual tank. Each tank was a plastic container that contained two interfolded paper towels, one moist and one dry. *Leiopelma hochstetteri* also had a bowl of water in the container. The containers were cleaned and the towels were changed weekly.

Temperature

In summer, the day temperature was 15°C and the night temperature was 11°C; this 4°C drop happened over 2 h. In winter, both day and night temperatures were 1–2°C lower.

Humidity/watering

Artesian spring water that had been twice filtered through ceramic micron-sized filters was used. The large breeding tanks had an automatic misting system while the plastic boxes were manually moistened. Moisture levels were monitored by carers

Lighting

Low-intensity (15 watt) fluorescent light was angled towards the ceiling to simulate moonlight. Low-heat white or fluorescent light was used to simulate daylight. Lighting was maintained on a photoperiod that roughly reflected the seasons.

Hygiene protocols

LabServ Nitril powder-free gloves were used at all times when handling frogs and equipment. The containers were cleaned out using only water, and no disinfectants were allowed on any equipment that would contact the frogs. The full hygiene protocols adhered strictly to the instructions of the DOC Native Frog Husbandry manual (Webster 2002).

Observations/handling

Each container was picked up daily to view the frog through the container; thus, every animal was sighted every day. No animals were touched except for a monthly weight check; this included during cleaning of the container each week. Snout-vent length was measured once every 6 months. If a frog was sick, observations increased to twice a day and treatment was given to the frogs if necessary.

Feeding

Individuals were fed weekly with a variety of invertebrates—crickets, fruit flies, houseflies and moth larvae.

A3.2 University of Otago

Contact

Dr Phil Bishop, academic staff member, Department of Zoology (email: phil.bishop@stonebow.otago.ac.nz).

Species

Leiopelma pakeka ex CU, wild-caught *L. archeyi*, and *Litoria* spp. are each kept in separate rooms, and all the *Leiopelma* are housed individually. Some chytrid-positive specimens are present. Only *L. pakeka* are referred to in this study.

Group housing

Both the group tanks and individual housing are in a designated frog room in the animal suite. The frog room is wired to an alarm that will sound if any of the temperature, lighting, watering or humidity systems fail. At the end of 2008, they will start to house a maximum of six *L. pakeka* in group tanks. Each group tank is essentially a glass tank with a hole in the bottom and the top and half of the front made from stainless steel mesh. The floor is made of plastic floor tiles that have large holes in them, which are supported by PVC plumbing pipes. These tiles are covered with a layer of fibreglass mesh (1 mm × 1 mm) and a layer of pebble (3 cm deep). Covering this is another layer of fibreglass mesh, followed by a layer of sand (3 cm deep), another layer of fibreglass mesh, and a layer of topsoil

(3 cm deep). Several large pieces of schist and leaf litter are on the topsoil. The substrates were obtained locally from a garden supplier and autoclaved at 150°C for 3 h, following which they were allowed to air dry. The tank was then set up with the sprinkler system and allowed to acclimatise for 30 days. Leaf litter and dead wood were then introduced from Maud Island (thought to be free from amphibian chytrid) to seed the microfaunal component. After a week or two, fungi and many small soil invertebrates and dipterans were present.

Individual housing

At the time of this study, all frogs are being held individually in the frog room in clear, plastic, airtight lunch boxes (30 cm × 20 cm × 10 cm) with opaque coloured lids. There are no holes drilled into these and the collection of frog boxes is completely covered with a blackout curtain. The frogs are on two pieces of damp, unbleached paper towel that have been scrunched up to give frogs some topography. The frogs remain in these containers unopened for a week, as they are only physically checked or weighed once a week. When they are checked, any soiled paper towels and all faeces and uneaten food are removed.

Temperature

The frog room is accurately temperature controlled. The temperature varies from 12°C min. and 14°C max. in winter, to 14°C min. and 16°C max. in summer.

Humidity/watering

Water is filtered through 2 µm and allowed to stand for more than 48 h. It is then attached to a misting system supplied by Ecologic (Rainmaker Misting System Kit™). It rains for 1.5 min every 12 h in the group frog tanks. The relative humidity (RH) of the room is around 60% and is expected to exceed 85% in the actual tanks. The watering in the individual tanks is manually monitored.

Lighting

Fluorescent tubes are used to light the frog room. During the summer, these ramp up for an hour from 0% at night to a maximum of 10% to simulate dawn. They then remain at 10% for 11 h before ramping down for an hour to 0% to simulate dusk. This regime is adjusted by decreasing the total amount of daylight time to roughly simulate seasonal variance. There is no natural light source.

Hygiene

Everything that goes into the tanks (except live insects) is sterilised at 150°C for 3 h. Rubber gloves (LabTex Plus Powder-free Multi-Purpose Laboratory) are used for handling everything, including the frogs. The lab scales and measuring equipment are permanently kept in the frog room.

Observations/handling

Leiopelma pakeka are observed once a week, and *L. archeyi* 2–3 times a week. Frogs are examined visually *in situ* and their boxes are cleaned around them. They are weighed once a month and snount-vent length is measured twice a year. Individuals are handled when being moved from one box to another during experiments or treatments.

Feeding

Leiopelma pakeka are fed once a week, usually with five very small crickets (< 6 mm long). This diet is supplemented with wax moth larvae, houseflies, locusts or fruit flies once a month, during which time the number of crickets per frog is reduced to three

A3.3 Auckland Zoo

Contact

Andrew Nelson, Team Leader, NZ Fauna (email: Andrew.Nelson@aucklandcity.govt.nz).

Species

Leiopelma archeyi from both the Coromandel and Whareorino populations, which are not mixed. All individuals included in this study were ex University of Canterbury; however, they have recently acquired some *L. archeyi* that were wild-caught form the Whareorino population.

Group housing

Up to eight frogs are housed in each enclosure, but this maximum may be increased to ten as necessary. Enclosures are kept in a purpose-built frog house, within which are two separate rooms that have the same watering/humidity and temperature regimes (as outlined below). The enclosures have three sides of glass and a fourth side that has glass on the bottom half and sliding doors on the top half for access. These doors are made from untreated pine that has been well covered by black enamel paint and aluminium mesh; silicon-based glue was used in their construction. All individuals included in this study were kept on natural substrate. However, in early 2007 all the Whareorino population group housing substrate was changed to dampened commercial Hygenex paper towels that are changed weekly. All Whareorino frogs are kept in one room (room 2), while the Coromandel frogs are kept in a different room (room 1) on natural substrate, which consists of commercially sourced sand and gravel that have been boiled for 1 h; commercially bought palm peat made up with boiling water; and soil substrate with leaf litter from Tapu (Coromandel), which has been sun dried for 3 months (some soil was baked at 200°C for 1 h before being dried in the sun for 3 months). The substrate is never changed but is spot cleaned. Substrate tanks have enclosure 'furniture' sourced from either Tapu or Auckland Zoo, which includes punga logs, broken terracotta pots and drip tray shelters.

Individual housing

Individual housing is currently used for quarantined or sick animals. Frogs are held in plastic Pet-pals containers that contain dampened commercial Hygenex paper towels, which are changed weekly. The tops are covered with Glad wrap to ensure the correct humidity is maintained and containers are kept within the same type of glass terrarium as is used for group housing but without lids. These animals are held in the same room as the Whareorino frogs (room 2).

Temperature

The frog house temperature is kept between 11°C and 15°C. Once a week, the temperature is reduced over 3 consecutive days to 8°C to help reduce bacterial load. There is a monitored temperature alarm system, which activates an alarm if the temperature goes above 15°C.

Lighting

In room 1, a standard fluorescent light is used during the day in addition to natural west-facing daylight filtered by glass. In room 2, a 15-watt shaded bulb is used during the night and an additional 15-watt unfiltered incandescent bulb is used during the day. Both rooms are on 12-h day/night cycles. In addition, both rooms are exposed to reptile/amphibian Acadia™ compact light 5 minutes a week. In early 2007, the fluorescent light in room 1 was discontinued.

Watering/humidity

The enclosures are watered with reverse osmosis water for 5 min four times a week, using hand sprayers that put 3–10 mL in each terrarium, the exact amount depending on how dry the soil is. Since early 2007, this system has been replaced by a manual turn-on irrigation system that uses Nylex irrigation spouts and a hose in each terrarium. There is also a small pot plant drip tray in each enclosure, which is filled with water so the frogs can soak themselves. Both rooms aim for 100% humidity, but this can vary down to 80%. The monitored humidity alarm is set for 60%. The water was changed to filtered instead of reverse osmosis water in early 2007.

Hygiene

Medishield (chlorhexidine) hand disinfectant is used to wash hands, and rubber gloves (Med X synthetic) are used when handling all items and are changed between enclosures. LabServ Nitril powder-free gloves have been used for handling frogs since mid-2006, as the Med X synthetic gloves seem to lather up when wet. Ammonia bleach is used to disinfect items that have been used in the enclosure and reverse osmosis water is used to rinse these items to ensure there is no residue.

Observations/handling

The individually housed frogs are weighed once per week. Colony Whareorino frogs are handled and weighed a maximum of every 6–8 weeks. Colony Coromandel frogs have also been on that regime, but are now being handled once every 6 months to minimise disturbance for breeding. The tanks are observed three times a week and any frogs seen are noted.

Feeding

The frogs were originally fed once a week. However, the colony frogs were changed to twice a week 1 year ago and the individual frogs were changed to this regime 3 months ago. Under this new regime frogs are given the same amount of food—just divided into two feedings. Each group-housed frog is fed six wax moth larvae (dusted with Miner-All Outdoor supplement at every feed

and Herptavite on the first feeding of each month), two or more house flies and 20 or more fruit flies; crickets are fed out to colony enclosures on Fridays. Each individually housed frog is fed six wax moth larvae, four house flies, and 20–30 fruit flies, all of which are dusted with Miner-All Outdoor supplement or Herptavite; four crickets < 5 mm are fed on Fridays.

A3.4 Hamilton Zoo

Contact

Kara Goddard, zookeeper (email: kara.goddard@hcc.govt.nz).

Species

Leiopelma hochstetteri ex University of Canterbury.

Group housing

All frogs are kept in an outdoor enclosure unless they are sick or in quarantine. The enclosure is wooden, with Perspex-lined walls and plastic liner in the pools and streams. The waterways contain small, smooth gravel as well as rocks, soil and leaf litter. The enclosure has a roof, and there are native trees on one side of the enclosure and another enclosure on the other side; however, there is natural patchy sunlight on the ground inside. There are three habitat cells in the enclosure, each of which is 1.7 m × 2 m and houses a maximum of 15 individuals. The substrate is gravel, rocks, screened topsoil and leaf litter, all of which were purchased or obtained on site. All rocks and gravel were rinsed and/or scrubbed and then thoroughly dried, and all soil, logs and leaf litter was dried in a hot shed (over 20°C ambient temperature) for 2 weeks before being used in the enclosure.

Individual housing

Individual housing is currently only used for quarantine or disease isolation purposes for a limited amount of time. The housing is a plastic container ('terrarium') that contains moistened, unbleached paper towels. It is sprayed with filtered water and paper towels are changed twice a week. The temperature is kept as cold as possible with an air conditioner. The terraria are kept in a darkened room with only a small amount of light allowed in during daylight hours.

Temperature

Natural Hamilton conditions.

Lighting

Natural Hamilton conditions.

Water/humidity

Natural Hamilton conditions. There is a stream system and seepage in each habitat cell, and irrigation in the roof, which comes on once or twice a day at variable times and for different lengths of time.

Hygiene

Rubber gloves (Lab Serv Nitril, powder-free) are used for handling frogs and equipment. A variety of disinfectants are used for cleaning equipment and bench tops depending on the stock available: Virkon (1%) solution, Trigene (1%) solution, bleach (5%) solution and clear methylated spirits.

Observations/handling

Each group-housed frog is weighed, measured and examined every 2 months. Individually housed frogs are checked daily and weighed weekly. The outdoor enclosure is checked daily for any problems and a nocturnal frog count survey is done every 2 weeks.

Feeding

All individuals are fed crickets less than 5 mm, wax moth larvae (small) and *Drosophila* three times a week.

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Chapter #	Details of publication(s) on which chapter is based	Nature and extent of the intellectual input of each author	I confirm the candidate's contribution to this paper and consent to the inclusion of the paper in this thesis	Name :	Signature
TWO	Shaw, S.D., and A. Holzapfel. 2008. Mortality of New Zealand native frogs in captivity. Department of Conservation Science and Technical Publishing, Wellington. 1-30.	Avi Holzapfel contributed to the report by assistance with analyzing data, organization of the report, and major editing .	Yes that is correct.	Avi Holzapfel	5/10/2011

Chapter 3: Designing a diet for captive native frogs from the analysis of stomach contents from free-ranging *Leiopelma* spp.

Preamble

The diet of captive *Leiopelma* was often discussed among the facilities that held leiopelmatids and the Department of Conservation Native Frog Recovery Group as a topic of concern due to its suspected role in contributing to metabolic bone disease. Eggers (1998), in an MSc thesis, had examined the stomach contents and Kane (1980), in an Honours thesis, had examined the faecal content of native frogs, both in an effort to describe the invertebrates eaten by wild native frogs. Both studies had useful findings but had made no dietary recommendations for the captive facilities to implement. As I had access to the largest collection of stomach contents ever obtained from wild *Leiopelma* spp., the opportunity to analyse these contents and make recommendations on how to improve the captive diet aligned well with my investigation into metabolic bone disease (discussed in Chapter Four).

The wild frogs used in this study were *Leiopelma archeyi* and *Leiopelma hochstetteri* that had fallen into pitfall traps aimed at invertebrates during a long-term Department of Conservation project. This chapter analyses stomach contents of these frogs but subsequent chapters use these same frogs for collecting other baseline information that would have otherwise been logistically impossible to obtain due to the critically endangered conservation status of *L. archeyi*. The aims of this chapter were to:

1) describe the invertebrate fauna ingested by free-ranging native frogs;

2) compare this to the diet of captive frogs; and

3) make recommendations on how to improve the captive diet, based on the assumption that the wild diet was superior.

This chapter is the original manuscript as published in a peer-reviewed journal: Shaw S.D., Skerratt L.F., Kleinpaste R., Daglish L., Bishop P.J., (2012). Designing a diet for captive native frogs from the analysis of stomach contents from free ranging *Leiopelma*. New Zealand Journal of Zoology 39: 47-56.

My contribution: 90% (detailed in co-author publication release form at the end of this chapter).

New Zealand Journal of Zoology
iFirst, 2012, 1–10

Designing a diet for captive native frogs from the analysis of stomach contents from free-ranging *Leiopelma*

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(*Received 12 May 2011; final version received 30 August 2011*)

Diets for captive amphibians are often inadequate and lead to poor health. To determine the natural diet of two New Zealand frog species, we analysed the stomach contents of 16 Archey's frogs (*Leiopelma archeyi*) from the Moehau Range of the Coromandel Peninsula and nine Hochstetter's frogs (*Leiopelma hochstetteri*) from the Moehau Range of the Coromandel Peninsula, the Hunua Ranges and Maungatautari. These specimens were obtained as by-catch from invertebrate pitfall traps from 2002 to 2008. Both species ate a wide range of invertebrates including springtails, mites, ants, parasitic wasps, amphipods and isopods. *Leiopelma archeyi* also ate snails. The mean ratio of maximum prey size ingested to snout–vent length in *L. archeyi* was 0.31 (range 0.16–0.5), and in *L. hochstetteri* was 0.42 (range 0.21–0.75). We suggest a reformulated captive diet based on the species and size of invertebrates ingested in the wild. This diet may assist in the prevention of metabolic bone disease.

Keywords: Coromandel Peninsula; diet; Hunua Range; *Leiopelma archeyi*; *Leiopelma hochstetteri*; Maungatautari; metabolic bone disease; Moehau Range; New Zealand; pitfall traps; stomach contents

Introduction

Archey's frog (*Leiopelma archeyi*) and Hochstetter's frog (*Leiopelma hochstetteri*) are two of four extant *Leiopelma* species in New Zealand. *Leiopelma archeyi* holds the number one position of the Evolutionarily Distinct and Globally Endangered (EDGE) list of amphibians, while *L. hochstetteri* is at number 38 (Edge of Existence, http://www.edgeofexistence.org [accessed 3 August 2010]). In addition, the New Zealand Threat Classification for *L. archeyi* is 'Nationally Vulnerable', whereas *L. hochstetteri* is considered 'At Risk: Declining' (Newman et al. 2010).

The Department of Conservation Native Frog Recovery Plan recommended captive breeding as one mode of conservation if wild populations were under threat (Newman 1996). In 1999, chytridiomycosis was thought to be the cause of a decline in the Coromandel *L. archeyi* population and it was suggested that all native frogs were at risk (Bell et al. 2004). As a result, captive colonies were started at the University of Canterbury in 2000, and were later transferred to Auckland Zoo (*L. archeyi*) and Hamilton Zoo (*L. hochstetteri*) in 2006. The University of Otago also acquired 12 *L. archeyi* for research on chytridiomycosis in 2006 during an emergency translocation (Bishop et al. 2009).

Unfortunately, these captive populations have had high mortality rates and little breeding success. Although the causes of mortality

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ISSN 0301-4223 print/ISSN 1175-8821 online

http://dx.doi.org/10.1080/03014223.2011.619989
http://www.tandfonline.com

from 1999 to 2006 were variable, most were attributed to secondary bacterial infections (Shaw & Holzapfel 2008). From late 2007, metabolic bone disease (MBD) from a combination of inadequate calcium intake, lack of UVB light, and exposure to fluoridated water is thought to have been the major cause of morbidity and mortality in all institutions (Shaw et al. 2009). This finding highlighted the importance of nutrition in the captive husbandry of these frogs and the need to look at their normal diet in the wild.

One early field observation from the Coromandel reported that stomach contents from an unspecified number of *L. hochstetteri* contained various beetles, dragonflies and a fresh-water crayfish (Stephenson & Stephenson 1957). A report by Eggers (1998) described the stomach contents of eight free-ranging leiopelmatid frogs (species unspecified) in Whareorino Forest caught in invertebrate pitfall traps. The main finding of her study was that the highest total numbers of invertebrates found were from the Orders Acari, Collembola, Amphipoda and Coleoptera, with lesser amounts of invertebrates from the Aranae, Diplopoda, Diptera, Gastropoda, Hemiptera, Hymenoptera, Isopoda and Pseudoscorpionida. The ratio of the largest invertebrate eaten to the frog's snout–vent length (SVL) ranged from 0.20 to 0.62 (Eggers 1998). An earlier report from Kane (1980) examined the faeces from both *L. archeyi* ($n = 35$) and *L. hochstetteri* ($n = 24$) collected over 4 years in the Coromandel region, and identified invertebrates from the Orders Acari, Amphipoda, Aranae, Coleoptera, Diptera, Gastropoda and Hymenoptera. Of these, amphipods occurred in *L. hochstetteri* significantly more often than *L. archeyi*, and only *L. archeyi* were found to have eaten mites (Kane 1980). A recent study classifying the trophic position of *L. hochstetteri*, reported that they eat primarily terrestrial invertebrates, although the exact invertebrates eaten were not determined (Najera-Hillman et al. 2009). As *L. archeyi* is terrestrial, and *L. hochstetteri* is semi-aquatic, it is likely that they eat different prey species.

From 2002 to 2008, the New Zealand Department of Conservation (DOC) and EcoQuest (a New Zealand foundation delivering study abroad programmes) set invertebrate pitfall traps in three locations on the north-eastern North Island: the Moehau Ranges, the Hunua Ranges and Maungatautari. The purpose of these traps was to determine how mammalian pest control affected forest invertebrate abundance and diversity (Rate 2009; R. Brejaart, EcoQuest, pers. comm. 2011). As the pitfall traps were in known native frog habitat, protective covers were suspended above the traps to prevent frogs hopping into the opening. It was not thought to be possible to exclude frogs from crawling through side openings without potentially affecting invertebrate catch rates and compromising the research, and as a result of this a small number of frogs were captured and died within minutes in the traps (O. Overdyck, DOC, pers. comm. 2010). This provided an opportunity to examine the stomach contents of free ranging *Leiopelma* to allow comparison with earlier findings and to assist with the re-formulation and improvement of captive diets.

Methods

All frogs were collected as an accidental by-catch in invertebrate pitfall traps set in other studies (Baber et al. 2006; Rate 2009; R. Brejaart, EcoQuest, pers. comm. 2011). Pitfall traps were placed at 90–100-m intervals along transects and checked monthly from 2002 to 2008. Frogs were found throughout the seasons and the years. The traps contained either 50–150 ml of 30–60% ethylene glycol or 100 ml of 10% sodium benzoate as a temporary preservative for the invertebrates. When frogs were found, they were transferred to individual containers with 70% ethanol. In total, 24 *L. archeyi* and nine *L. hochstetteri* were caught and died in pitfall traps.

Frogs were dissected 1–5 years after collection using a standard frog necropsy protocol (Rose 2007) with a few modifications in order

to collect samples for future genetic work and for cryopreservation. SVL was measured to the nearest tenth of a millimetre using electronic callipers (J. Germano unpubl. data) and individuals classified as an adult, subadult or juvenile based on the SVL classification scheme of Bell (1978). Post mortems were performed on eight adult, seven subadult and one juvenile *L. archeyi*, as well as four adult and five subadult *L. hochstetteri*. Two adult specimens were too decomposed and six juvenile specimens were too small to be necropsied for the original purposes of the study.

The entire contents of the stomach were removed and placed into 70% ethanol. Invertebrate food items, entire or partially digested, were measured and identified to class, order or family and, depending on their condition, to more detailed taxonomic levels. When parts of specimens were identified, the approximate original sizes of the invertebrates were estimated by comparing the fragments with whole specimens of similar life stages. Measurements were taken to the nearest millimetre for the larger specimens and to the nearest 0.5 mm for the smallest. Identification to family or genus level was achieved by comparing the specimens with invertebrates held in a reference collection (R. Kleinpaste unpubl. data). Ants and beetles were identified following taxonomic keys (Klimaszewski & Watt 1979; Don 2007).

The percentage of invertebrates belonging to each order was determined. The number of times an invertebrate order was found in the stomach was counted and given as the frequency of that order in each frog species. Using a Fisher's exact test with WinPepi Version 11.4 (http://www.brixtonhealth.com/pepi4windows.html), comparisons were made between both *L. archeyi* and *L. hochstetteri*, as well as between age classes within species.

Results

All frog specimens had stomachs completely full of invertebrates. In three *L. archeyi*, a seed, seed husk or a minor amount of decaying plant matter was found, and in one *L. hochstetteri* a leaf was found.

There were 148 individual invertebrates found in the stomachs of 16 *L. archeyi* and 63 in the stomachs of nine *L. hochstetteri*. The percentage of invertebrates belonging to each order out of the total number of invertebrates identified is given in Table 1 with family and genus listed if determined. In *L. archeyi*, the three most abundant orders were Collembola (springtails), Acari (mites) and Hymenoptera (ants, parasitic wasps), whereas in *L. hochstetteri*, they were Amphipoda (hoppers), Isopoda (slaters) and Acari (mites).

The percentage of *L. archeyi* and *L. hochstetteri* stomachs that contained a type of invertebrate (by order) is given in Table 2. In *L. archeyi*, the three most frequently occurring orders were Acari, Hymenoptera and Collembola, whereas in *L. hochstetteri*, they were Isopoda, Amphipoda and Aranae (spiders).

Some orders were more common in the subadults than the adults, or more common in one of the frog species. In *L. archeyi*, six of the seven frogs that ate Collembola were subadults ($P = 0.01$; odds ratio = 42; 95% CI 2–2195). Gastropoda (snails) were found in more *L. archeyi* than *L. hochstetteri* ($P = 0.02$; odds ratio = 15; 95% CI 86–260).

The maximum sizes of invertebrate prey found in the stomachs of an adult, subadult and juvenile *L. archeyi* were 10, 12 and 2 mm, respectively. The mean ratio between maximum size of invertebrate prey and SVL in *L. archeyi* was 0.31 (range 0.16–0.5). The maximum size of invertebrate prey in an adult *L. hochstetteri* was 22 mm and in a subadult 14 mm. The mean ratio between maximum size of invertebrate prey and SVL in *L. hochstetteri* was 0.42 (range 0.21–0.75).

Discussion

One limitation of our study was the small number of frogs available, with most specimens from one location. Although frogs were found in each season, the small numbers made

Table 1 Number of invertebrates in stomachs of *Leiopelma archeyi* and *Leiopelma hochstetteri*.

Invertebrate identification	Total number in *L. archeyi* stomachs (*n*)	Total invertebrates (%)	Total number in *L. hochstetteri* stomachs (*n*)	Total invertebrates (%)
Acari (mites)	19	13.0	5	7.9
Oribatid	9		3	
Trombiculid	7		1	
Anystidae	2		0	
Other	1		1	
Amphipoda (hoppers)	9	6.2	20	31.8
Aranae (spiders)	5	3.4	4	6.4
Dysdera crocata (slater spider)	1		0	
Pholcidae (daddy long-leg)	0		2	
Salticidae (jumping spider)	1		0	
Other	3		2	
Blattaria (cockroaches) *Celatoblatta*	0		1	1.6
Coleoptera (beetles)	8	5.5	3	4.8
Curculionidae (weevil)	3		0	
Scaphidiinae (shining fungus beetle)	1		1	
Other	4		2	
Collembola (springtails)	35	24.0	1	1.6
Dermaptera (earwigs)	1	0.7	0	0
Diplopoda (soft and hard millipedes)	6	4.1	4	6.4
Diptera (flies)	10	6.9	4	6.4
Tipulidae (crane fly)	5		1	
Therevidae (stiletto fly)	1		0	
Mycetophilidae (fungus gnats)	2		0	
Other	2		3	
Gastropoda (snails)	13	8.9	0	0
Geophilomorpha (centipedes) *Geophilus*	0	0	2	3.2
Hemiptera (bugs)	8	5.5	1	1.6
Aradidae	4		0	
Cicadidae	1		1	
Heteroptera, Miridae, Lygaeidae, other	3		0	
Hymenoptera	18	12.3	2	3.2
Formicida (ants), *Hypoponera*, *Amblyopone*, *Pheidole*, other	15		1	
Braconidae (parasitic wasp)	3		1	
Isopoda (slaters) *Porcellio scaber*, other	4	2.7	14	22.2
Lepidoptera (moths)	5	3.4	0	0
Tineoidea	1		0	
Other	4		0	
Opiliones (harvestman)	2	1.4	0	0

Table 1 (*Continued*)

Invertebrate identification	Total number in *L. archeyi* stomachs (*n*)	Total invertebrates (%)	Total number in *L. hochstetteri* stomachs (*n*)	Total invertebrates (%)
Orthoptera (ground weta), *Hemiandrus*	1	0.7	2	3.2
Pseudoscorpionida (false scorpion spider)	1	0.7	0	0
Thysanura (silverfish)	1	0.7	0	0
Total number identified	146		63	
Unidentified	2	1.4	2	3.2

seasonal comparisons not feasible. It is unlikely that larger numbers will ever be available, as these frogs are under strong protection and permits are not easily obtained. However, as our findings in general concur with previous studies, some robust conclusions seem possible. There are three findings that agree with Eggers (1998) and Kane (1980):

(1) Both frog species eat a broad diversity of food items.
(2) In *L. archeyi*, Acari and Collembola are more abundant than other orders.
(3) Amphipods were more frequently found in *L. hochstetteri* than *L. archeyi*, although the difference was not significant.

Our study has three additional findings:

(1) In *L. archeyi*, Collembola are found significantly more in subadults than adults. If small size of prey was the determining factor for this choice, it would be expected that mites, which are even smaller, would also make up a significant component of the diet in subadults. However, this was not the case; therefore, some other factor(s) may be driving this selection.
(2) Approximately 33% of *L. hochstetteri* stomachs contained mites, whereas previous studies found none. As these frogs were from the same region as Kane's study (Kane 1980), this may have been because more mites were available to frogs in the years we sampled, or perhaps stomach content analysis is more sensitive than faecal analysis at detecting small invertebrates.
(3) Significantly more *L. archeyi* (44%) ate gastropods than did *L. hochstetteri* (0%). Although Kane (1980) discovered evidence of gastropods in *L. hochstetteri* faeces, all his samples were from the Coromandel. The nine stomach content samples in this study were from the Coromandel, the Hunua Ranges and Maungatautari, and this indicates that not all *L. hochstetteri* habitats may be suitable for gastropods.

As part of a study of MBD in captive *Leiopelma*, the supplemented captive diet of *L. archeyi* at Auckland Zoo (Shaw & Holzapfel 2008) was analysed for a variety of components (S. Shaw unpubl. data). Although the diet appeared to actually contain an unacceptably high calcium to phosphorus ratio (Ca:P) of 5:1 (1.5:1.0 being the goal according to Wright 2001), it is very unlikely that the frogs were actually ingesting a diet with that ratio for two main reasons:

(1) The invertebrates being fed out had naturally very poor Ca:P ratios (Anderson 2000; Finke 2002). The artificially high ratio was dependent on the majority of the calcium supplementation powder staying on the insects and being eaten by the frogs before it had been removed by the insect, which may occur within minutes to

Table 2 The percentage of *Leiopelma archeyi* and *Leiopelma hochstetteri* stomachs that contained a type of invertebrate (by order).

Invertebrate identification	Total number of all *L. archeyi* stomachs the invertebrate identified in $n=16$	Frequency (%)	*L. archeyi* adults only, $n=8$	Frequency (%)	*L. archeyi* subadults only, $n=7$	Frequency (%)	Total number of all *L. hochstetteri* stomachs the invertebrate identified in $n=9$	Frequency (%)	*L. hochstetteri*, adults only, $n=4$	Frequency (%)	*L. hochstetteri*, subadults only, $n=5$	Frequency (%)
Acari (mites)	10	62.5	4	50.0	5	71.4	3	33.3	0	0	3	60.0
Amphipoda (hoppers)	5	31.3	2	25.0	3	42.9	6	66.7	3	75.0	3	60.0
Aranae (spiders)	4	25.0	2	25.0	2	28.6	4	44.4	1	25.0	3	60.0
Blattaria (cockroaches)	0	0	0	0	0	0	1	11.1	1	25.0	0	0
Coleoptera (beetles)	6	37.5	3	37.5	3	42.9	3	33.3	1	25.0	2	40.0
Collembola (springtails)	7	43.8	1	12.5	6	85.7	1	11.1	0	0	1	20.0
Dermaptera (earwigs)	1	6.3	0	0	1	14.3	0	0	0	0	0	0
Diplopoda (soft and hard millipedes)	3	18.8	2	25.0	1	14.3	4	44.4	1	25.0	3	60.0
Diptera (flies)	6	37.5	3	37.5	3	42.9	4	44.4	2	50.0	2	40.0
Gastropoda (snails)	7	43.8	4	50.0	2	28.6	0	0	0	0	0	0
Geophilomorpha (centipedes)	0	0	0	0	0	0	2	22.2	1	25.0	1	20.0
Hemiptera (bugs)	6	37.5	4	50.0	2	28.6	1	11.1	1	25.0	0	0
Hymenoptera (ants and parasitic wasps)	8	50.0	4	50.0	3	42.9	2	22.2	1	25.0	1	20.0
Isopoda (slaters)	4	25.0	3	37.5	1	14.3	7	77.8	4	100.0	3	60.0
Lepidoptera (moths)	4	25.0	3	37.5	1	14.3	0	0	0	0	0	0
Opiliones (harvestman)	2	12.5	1	12.5	1	14.3	0	0	0	0	0	0
Orthoptera (ground weta)	1	6.3	1	12.5	0	0	2	22.2	1	25.0	1	20.0
Pseudoscorpionida (false scorpion spider)	1	6.3	1	12.5	0	0	0	0	0	0	0	0
Thysanura (silverfish)	1	6.3	1	12.5	0	0	0	0	0	0	0	0

hours (Wright 2001; Li et al. 2009). Observations have shown that not all prey is eaten within the first 24 h (N. Kunzmann, Auckland Zoo, pers. comm. 2010).

(2) The frogs were housed in a group tank, so it is possible that not all frogs had equal access to supplemented food.

Many of the invertebrates found in the stomachs of free-living *Leiopelma* have much higher calcium values on percentage dry matter (D.M.) basis than other invertebrates often fed to *Leiopelma* in captivity such as crickets (Orthoptera), houseflies and fruitflies (both Diptera). For example, free-living Isopoda, Gastropoda, Diplopoda and Thysanura have calcium values of 0.8%, 1.8% (flesh portion)/ 28.3% (shell portion), 16.8% and 0.4% D.M. respectively, in comparison with 0.2% and 0.1% D.M. of Orthoptera and Diptera, respectively (Reichle et al. 1969; Donoghue & Langenberg 1996). Although the shell of Gastropoda has a higher calcium content than the soft body portion, other invertebrates are likely to have no difference in calcium if the exoskeleton, wings and legs are removed as chitin contains negligible amounts of calcium (Studier & Sevick 1992; Densmore & Green 2007). Our study shows that captive *Leiopelma* are being fed a diet that does not resemble their natural diet and is probably too low in calcium. As there is still little information on nutritional requirements of amphibians, it is difficult to know how to substitute a natural diet safely with laboratory bred invertebrates and calcium supplements across a range of amphibian species (Young 2003). Future studies conducting nutritional analyses on individual prey items found in New Zealand would be helpful so that captive institutions have more information on mineral, vitamins and fat content when formulating their own diets.

We suggest that a re-formulated captive diet for *L. archeyi* and *L. hochstetteri* subadults and adults, based on our findings, as well as those of Kane (1980) and Eggers (1998), may decrease the reliance on vitamin and/or mineral supplements. New prey items should be introduced for palatability trials, e.g. Gastropoda to *L. hochstetteri*. We recommend using the average maximum prey size to the frog's SVL ratio as the basis of deciding the optimal size range of prey fed out, but allowing smaller numbers of larger prey items to be fed out as this ratio may vary. Using this ratio should ensure that the prey size mimics that which is eaten by free-living frogs of varying age, as the nutritional value of some invertebrates change with age and size (Finke 2002; Donoghue 2006). The percentage of invertebrates eaten by order is a useful guide to diet composition, but may not take into account the difference in size among the prey items and therefore their contribution in terms of the total volume of the diet. Using frequency of presence of an invertebrate within stomachs to formulate a captive diet may assist in including the less numerous, but important and larger invertebrates.

Several examples of captive diets following the above guidelines have been created (Appendix). These diets give an example of the type, amount and size of invertebrate recommended for feeding to one adult or sub-adult native frog weekly, based on the stomach contents of free-living individuals and how often the frogs defecate in captivity (P. Bishop pers. obs).

We have not adequately addressed the dietary requirements of juvenile frogs, as we were only able to examine one juvenile in this study. Froglet nutrition is an area that needs further study and will be important when froglets are produced in captivity. There are preserved juveniles held by DOC that may be available for this purpose. This natural diet, in conjunction with addressing other factors shown to cause MBD such as the amount of UVB received and fluoride exposure (Wright & Whitaker 2001; Young 2003; Shaw et al. 2009), would likely see a decrease, if not complete elimination, of new cases of MBD, and possibly an improvement in the captive breeding of healthy frogs. One way to help address this aim would be to house frogs in outdoor

enclosures with mesh large enough to allow invertebrates and UVB light through. We acknowledge that this type of captive diet may be costly in terms of labour and direct costs, but in light of the conservation and phylogenetic significance of these species, a complex captive diet simulating a natural one is essential.

Acknowledgements

Funding was provided by the New Zealand Veterinary Association Wildlife Society Marion Cunningham Grant. The authors would like to thank Gribbles Veterinary Laboratory, Auckland, for the use of their facility space and microscales. We also thank the Department of Conservation staff and EcoQuest staff and volunteers who set and checked pitfall traps, for their time and effort. Thanks to Peter West, Nicole Kunzmann, Natalie Clark, Tanya Shennan, Andrew Nelson, Ian Fraser, Michelle Whybrow and Lana Canzak—all native frog zookeepers at Auckland Zoo—who spent long hours collecting and breeding new invertebrates to feed out and for their support in changing the captive diet. Thanks to John Potter, veterinarian at Auckland Zoo for assistance and support with diet analyses. Many thanks to the Māori iwi for their support of research and conservation of native frogs: Hauraki Maori Trust Board, Mataora No 1 & 2 Block Inc, Moehau Nga Tangata Whenua, Ngāti Hei Trust, Ngāti Huarere, Ngāti Maru ki Hauraki Inc, Ngāti Pāoa Trust, Ngāti Pūkenga ki Waiau Society, Ngāti Rāhiri Tumutumu, Ngāti Rongo U Charitable Trust, Ngāti Tai Umupuia Te Waka Totara Trust, Ngāti Tāwhaki, Ngāti Whanaunga, Te Kupenga O Ngāti Hako Inc, Te Patukirikiri Iwi Inc, Te Rūnanga O Ngāti Pu, Te Rūnanga a Iwi o Ngāti Tamaterā and Te Uringahu Ngāti Maru Manaia.

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Appendix A: An example of the type, amount, and size of invertebrate that should be fed weekly to one frog (*Leiopelma archeyi* or *Leiopelma hochstetteri*, adult or subadult) based on stomach contents of free-living individuals.

Invertebrate order	Number (and size range in mm) to feed out to an adult *L. archeyi*[a]	Number (and size range in mm) to feed out to a subadult *L. archeyi*	Number (and size range in mm) to feed out to an adult *L. hochstetteri*	Number (and size range in mm) to feed out to a subadult *L. hochstetteri*
Acari (mites)	2.0 (0.5–2)	3.0 (0.5–2)	0	1.6 (0.5–2)
Amphipoda (hoppers)	0.5 (7–10)	2.0 (3–9)	4.0 (3–8)	5.0 (2–9)
Aranae (spiders)	0.8 (3–7)	0.5 (3–5)	0.5 (5)	1.2 (3)
Blattaria (cockroaches)	0	0	0.5 (12)	0
Coleoptera (beetles)	0.8 (2)	1.2 (2)	0.5 (4)	0.8 (4)
Collembola (springtails)	1.0 (2–4)	8.0 (1–4)	0	0.4 (3)
Diplopoda (soft and hard millipedes)	0.5 (8–12)	1.0 (1–5)	0.5 (5–14)	1.2 (5–14)
Diptera (flies)	1.2 (1.5–9)	1.2 (1.5–9)	1.0 (6–14)	0.8 (1.5–6)
Gastropoda (snails)	2.0 (1–4)	1.6 (1–4)	0	0
Hemiptera (true bugs)	1.0 (1–5)	1.0 (1–5)	0	0
Hymenoptera (ants and parasitic wasps)	2.8 (1–4)	2.0 (1–4)	0.5 (6)	0.4 (3)
Isopoda (slaters)	0.8 (4–7)	0.2 (2)	5.0 (4–14)	1.6 (2–4)
Lepidoptera (moths)	1.0 (1.5–12)	0.2 (1.5–12)	0	0
Opiliones (harvestman)	0.2 (3)	0.2 (3)	0	0

[a]The number to feed out was derived by dividing the total number of that invertebrate found in that particular group of frogs by the total number of frogs in that group.

Consent of Authors for previous published document.

Chapter #	Details of publication(s) on which chapter is based	Nature and extent of the intellectual input of each author	I confirm the candidate's contribution to this paper and consent to the inclusion of the paper in this thesis	Name :	Signature
THREE	Shaw, S.D., L.F. Skerratt, R. Kleinpaste, L. Daglish, and P.J. Bishop. 2011 Designing a diet for captive native frogs from the analysis of stomach contents from free-ranging Leiopelma. New Zealand Journal of Zoology 39:47-56	Lee Skerratt contributed to the MS by assistance with analyzing of results; major editing of the MS, and assistance with the rebuttal.	Yes that is correct.	Lee Skerratt	
		Ruud Kleinpaste contributed to the MS by identifying all the invertebrates and editing the methods section where it pertains to invertebrate indentification..	Yes that is correct.	Ruud Kleinpaste	
		Lisa Daglish contributed to the MS by assisting with permits and obtaining samples; provding information for the methods section where it pertains to the pitfall traps and minor editing of the methods and introduction.	Yes that is correct.	Lisa Daglish	
		Phillip Bishop contributed to the MS by major editing of the MS.	Yes that is correct.	Phillip Bishop	

Chapter 4: Fluorosis as a probable factor in metabolic bone disease in captive New Zealand native frogs (*Leiopelma* species)

Preamble

Metabolic bone disease is the most common disease of captive frogs. These findings have global relevance to frog conservation efforts, as in many cases the only proven intervention against chytridiomycosis is to bring frogs into captivity.

The overall aims of this chapter were to:

1) determine the prevalence of metabolic bone disease (MBD) in captive *Leiopelma archeyi* and *Leiopelma hochstetteri*;
2) diagnose the aetiology of the disease; and
3) make recommendations for prevention.

This investigation was exhaustive involving data from three captive facilities in two frog species and collaboration with three other diagnostic facilities. Chytridiomycosis was not identified as a cause of death in any captive cases and I found mortality rates continued to be high for captive *L. archeyi* and *L. hochstetteri*. The following published paper describes how the high mortality was caused by MBD and that it was a complex, multi-factorial issue involving an imbalanced diet and a lack of exposure to ultraviolet-B light. It also details how exposure to fluoride in the water played a major role in the aetiology of MBD in these frogs, which was previously undescribed in amphibians. I have included Figure 5 after the published document to enable colour viewing of the histology.

This chapter is the original manuscript as published in a peer-reviewed journal: Shaw, S. D., Bishop, P. J., Harvey, C., Berger, L., Skerratt, L. F., Callon, K., Watson, M., Potter, J., Jakob-Hoff, R., Goold, M., Kunzmann, N., West, P. & Speare, R. 2012. Fluorosis as a probable factor in metabolic bone disease in captive New Zealand native frogs (*Leiopelma* spp.) Journal of Zoo and Wildlife Medicine 43:549-565.

My contribution: 80% (detailed in co-author publication release form at the end of this chapter).

Journal of Zoo and Wildlife Medicine 43(3): 549–565, 2012

FLUOROSIS AS A PROBABLE FACTOR IN METABOLIC BONE DISEASE IN CAPTIVE NEW ZEALAND NATIVE FROGS (*LEIOPELMA* SPECIES)

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Abstract: This report describes the investigations into the cause and treatment of metabolic bone disease (MBD) in captive native New Zealand frogs (*Leiopelma* spp.) and the role of fluoride in the disease. MBD was diagnosed in *Leiopelma archeyi* and *Leiopelma hochstetteri* in 2008 at three institutions: Auckland Zoo, Hamilton Zoo, and the University of Otago. Most of these frogs had originally been held at the University of Canterbury for several years (2000–2004) but some were collected directly from the wild. Radiographs on archived and live frogs showed that MBD had been present at Canterbury, but at a lower rate (3%) than in the current institutions (38–67%). Microcomputed tomography showed that the femoral diaphyses of the captive frogs at Auckland Zoo had greater bone volume, bone surface, cross-sectional thickness, and mean total cross-sectional bone perimeter, which is consistent with osteofluorosis. On histology of the same femurs, there was hyperplasia, periosteal growth, and thickening of trabeculae, which are also consistent with skeletal fluorosis. An increase in fluoride levels in the water supply preceded the rise in the incidence of the above pathology, further supporting the diagnosis of osteofluorosis. Analysis of long-standing husbandry practices showed that ultraviolet B (UVB) exposure and the dietary calcium:phosphorus ratio were deficient when compared with wild conditions—likely causing chronic underlying MBD. To prevent multifactorial MBD in captive *Leiopelma*, the authors recommend increasing dietary calcium by incorporating into the captive diet inherently calcium-rich invertebrates; increasing exposure to natural or artificial (UVB) light; and using defluoridated water. Addressing these three factors at Auckland Zoo reduced morbidity, bone fractures, and mortality rates.

Key words: Amphibian, calcium, fluorosis, *Leiopelma*, metabolic bone disease, New Zealand, osteodystrophy, ultraviolet.

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INTRODUCTION

Understanding factors that affect the health of amphibians is important, as more than a third of species are threatened with extinction.[28] This includes the New Zealand native frog fauna comprised of four extant species that have the following IUCN threat classifications: *Leiopelma archeyi* (critically endangered), *Leiopelma hamiltoni* (endangered), *Leiopelma hochstetteri* (vulnerable), and *Leiopelma pakeka* (vulnerable).[11] Leiopelmatids are an archaic family of anurans with many unique features. They are all nocturnal, terrestrial direct developers, except for *L. hochstetteri*, which are semiaquatic.[3] They range in size from 25 to 47 mm snout–vent length[2] (SVL) and average 4–8 g in weight, depending on species, with *L. archeyi* being the smallest and *L. pakeka* the largest (P. Bishop, unpubl. data). In 1996, the Department of Conservation produced a Native Frog Recovery Plan[19] for the main purpose of halting any further declines of *Leiopelma* species

and the long-term goal of maintaining and enhancing existing genetic stocks.

Maintaining healthy captive collections of these species has been challenging. From 2000 to 2004, approximately 252 wild *Leiopelma* were collected and brought into captivity to Canterbury University for captive propagation and research. In late 2005–2006, the 154 remaining frogs from Canterbury University were split by species and sent to different institutions. All *L. archeyi* went to Auckland Zoo, *L. hochstetteri* to Hamilton Zoo, and L. pakeka went to the University of Otago, Dunedin and Zealandia (previously Karori Sanctuary) in Wellington. In December 2006, 16 additional *L. archeyi* obtained from the Whareorino forest were sent to Auckland Zoo and 12 to the University of Otago.[4] Captive mortality at Canterbury University from 2000 to 2005 was considered high, averaging 9% for *L. archeyi* and 12% for *L. hochstetteri* over the 5 yr.[24] The primary cause of mortality was usually unknown; the most common finding on histology was bacterial infection.[24] Clinical syndromes seen were skin blisters, keratitis, tetanic spasms, and weight loss.[21] Reproduction at all institutions was extremely poor—only two *L. archeyi* froglets were produced over 10 yr and neither survived to the subadult stage (S. Shaw, unpubl. data).[24]

In October 2007, three frogs at Auckland Zoo presented with asymmetrical jaws or open maxillary fractures. Metabolic bone disease (MBD) was suspected, as even slight mandibular deformities and tetany are suggestive of this disease.[36] Radiographs of deceased frogs from Canterbury University were taken and postmortem reports reviewed to determine if the disease had been present there and at what prevalence or if it was a new disease. Only three suspect cases were detected from the preceding 7 yr, indicating the clinical syndrome was not new, but also that the current cluster of three new cases was unusual. Subsequent pathological examinations confirmed the diagnosis and examination of husbandry revealed several potential risk factors: The diet was low in calcium and high in phosphorus; the frogs had no exposure to ultraviolet B (UVB) light or dietary vitamin D_3; and the water used at all institutions except Canterbury University contained fluoride.

Metabolic bone disease is one of the most commonly recognized nutritionally related disorders in captive amphibians,[36] but there is a paucity of detailed epidemiological studies in the literature.[9,14,36,38] Metabolic bone diseases, or osteodystrophies, are traditionally classified as rickets, osteomalacia, fibrous osteodystrophy, or osteoporosis. These are distinct morphologically but can occur in combination within the same individual.[32] The term MBD encompasses many etiologies, most of which are related to a deficiency of several nutrients or an imbalance of calcium, phosphorus, and vitamin D_3 or ingestion of another substance (fat-soluble vitamins, various minerals, oxalates, and fluoride) that interferes with the absorption or utilization of one of these compounds.[31,32,36] Amphibians may vary in their ability to use dietary vitamin D_3 instead of exposure to UVB, and little information is known regarding how much UVB is necessary.[1] In the nocturnal great barred frog (*Mixophyes fasciolatus*), it was found that UVB exposure had no significant effect on bone mineral content or skeletal histology, whereas percutaneous administration of calcium and vitamin D_3 resulted in adequate skeletal mineralization.[38] MBD was also reported in captive-bred mountain chicken frogs (*Leptodactylus fallax*) and it was suggested that hypocalcemia was the cause, but exposure to UVB was not discussed.[14] However, one recent investigation has shown that the addition of daily UVB significantly increased growth and skeletal development in captive metamorphosing Amazonian milk frogs (*Trachycephalus resinifictrix*).[33]

Fluorosis has not been reported in amphibians, but has been reported to cause toxicity in some aquatic organisms, as fluoride can be taken up directly from the water.[5,26,27] Toxicity in fish is dependent on species, size, water temperature, and water levels of fluoride, calcium, and chloride.[5] Adverse effects include growth inhibition, behavioral changes, tetany, anorexia, weight loss, embryonic death, and bone abnormalities.[5,6,27] Fluoride accumulates in bone by substitution of the hydroxyl and carbonate ions into the hydroxyapatite crystal structure, which can lead to impaired mechanical properties.[26,30] In mammals, the rate of this change in bone structure and therefore the degree of clinical disease is affected by the dietary intake of calcium and synthesis of vitamin D.[13,31]

Osteofluorosis was suspected to be the cause for the increased incidence of MBD observed in *L. archeyi* and *L. hochstetteri* in this study. Further investigations reported here, involving Auckland Zoo, the University of Otago, and Hamilton Zoo, were aimed at verifying the diagnosis of fluorosis, characterizing other factors associated with MBD, and exploring ways to monitor the treatment and recovery of the frogs.

MATERIALS AND METHODS

Initial investigations were aimed at determining the extent of MBD and confirming the diagnosis of osteofluorosis. This was then followed by an analysis for risk factors for MBD, osteofluorosis, and mortality and an assessment of the response of frogs to treatment of risk factors.

Extent of signs of MBD

Retrospective review of postmortem reports: Postmortem reports produced by Massey University were reviewed for mention of fractures. This included frogs from Auckland Zoo 2006–2008 (18 *L. archeyi*) and Canterbury University 2005–2006 (5 *L. archeyi* and 20 *L. hochstetteri*).

Skeletal radiography: Auckland Zoo: At the Auckland Zoo, plain radiographs were taken and assessed by the same veterinarian for bone density and presence of fractures. A free-standing X-ray machine (Acoma Diagnostic X-ray Unit VR 1020, Delta Building Blok A-11, Jl. Suryopranoto No. 1–9, Jakarta 10160, Indonesia), mammographic screen and cassette 18 × 24 cm (Kiran Screen CE0044; Kiran Medical Systems, Khar, Mumbai 400052, India) and mammography film (Fuji Medical Mammography film UM-MA 100NIF, Albany, Auckland, 0632, New Zealand) were used with an automatic processor (Model JP-33, Jungwan Precision Industries Ltd., 152–769 Guro-gu, Seoul, South Korea). Live frogs were placed in plastic damp specimen containers (diameter 8 cm) without lids in a normal resting position, and dead frogs were laid directly on the cassette. The cassette was portioned off with lead dividers and the ray coned to ¼ of the cassette. Whole-body dorsal–ventral views were taken. After an initial trial of settings, radiographs of adult frogs (24.5–35 mm SVL[2]) were taken at 54 kVp and 4.0 mAs, whereas for subadults (11–24 mm SVL) and juveniles (<11 mm SVL) radiographs were taken at 52 kVp /4.0 mAs to 52 kVp /3.2 mAs, depending on size. All live frogs were radiographed as part of the initial investigation in February 2008 ($n = 60$) and again 2 and 25 mo after treatment was instigated ($n = 58$ and $n = 25$, respectively). In addition, a random subset of these frogs both with and without previous fractures were x-rayed from each enclosure 6 and 13 mo posttreatment ($n = 17$ and $n = 16$, respectively). All dead frogs from 2008 onwards also had radiographs taken as part of the postmortem examination ($n = 37$).

University of Canterbury and wild frogs: At the Auckland Zoo, radiographs were also taken of *L. archeyi* ($n = 23$) and *L. hochstetteri* ($n = 15$) that died in captivity at the University of Canterbury from 1999 to 2004 that did not have a postmortem examination, and free-ranging *L. archeyi* ($n = 29$) and *L. hochstetteri* ($n = 12$) either found dead in the wild or accidentally caught in pitfall traps.[22]

University of Otago: The University of Otago Dental School radiographed the *L. archeyi* in the Department of Zoology holdings ($n = 12$) in October 2008 and then yearly. Frogs were placed in moist plastic bags and radiographs were taken with the use of dental film (Kodak Insight, Carestream Dental LLC, Atlanta, Georgia 30339, USA), a dental X-ray machine (Prostyle Intra, Planmeca Inc., Roselle, Illinois 60172, USA) and processed with an automatic developer (model All-Pro 2010, AllPro Imaging, Melville, New York 11747, USA). Exposure factors were 60 kVp/8 mAs.

Hamilton Zoo: The Hamilton Zoo radiographed most of the *L. hochstetteri* in their holdings in October 2008 ($n = 24$) and repeated this every 6 mo as part of a routine biannual health check. Frogs were placed in individual plastic bags and radiographs were taken with the use of dental film (Kodak Insight, Carestream Dental) and a Shimadzu mobile X-ray unit (Model MC 125–30) and processed manually (Kodak GBX developer and fixer, Carestream Dental). Whole-body dorsal–ventral views were taken with exposure factors of 60 kVp/20 mAs.

Rates of skeletal fractures of the frogs at each institution were calculated and a two-tailed Fisher's P test was used for comparison.

Diagnosis of osteofluorosis

Microcomputer tomography scans of femurs: Five femurs from Auckland Zoo *L. archeyi* that died in July and August 2008 (6–8 mo after starting calcium and vitamin D_3), and six from wild pitfall trap *L. archeyi* were dissected out and placed in 70% ethanol. All frogs were adults based on SVL.[2] Two of the captive frog femurs scanned had femoral folding fractures, one had a mild folding fracture, and two did not have any femoral fractures.

Femurs were scanned with the use of a Skyscan 1,172-μ CT scanner (X-ray voltage 49 kV, 0.5-mm aluminum filter; isotropic voxel size 14 μm) (Skyscan, Kartuizersweg 3B, 2550 Kontich, Belgium). After standardized reconstruction with the use of Skyscan NRecon software, the data sets were analyzed with Skyscan CT-analyzer software (CTAn, Skyscan). Volumes of interest (VOIs) were selected in three regions: at the proximal

end, middiaphysis, and distal end of each bone. The VOIs at the proximal and the distal ends were positioned 420 μm from where mineralized bone was first visible. In all regions, the height of the VOI was 140 μm.

t-tests were done to compare the weighted difference between mean bone measurements of femurs from the wild pitfall trap frogs and the Auckland Zoo frogs at the proximal epiphysis of the femur, middiaphysis, and the distal epiphysis in the following categories: bone volume, bone surface, cross-sectional thickness, and mean total cross-sectional bone perimeter.

Postmortem exams and histology: Postmortem examinations were performed routinely if a frog died at Auckland Zoo, and a variety of tissues were submitted for histological processing[23] to a veterinary pathology laboratory (Gribbles Veterinary Laboratories, Penrose 1642, Auckland, New Zealand). Slides were reviewed by a pathologist.

Femurs from 19 *L. archeyi* were also prepared for histology (including the 11 that had undergone CT scans and 7 from frogs that died at Auckland Zoo between September 2009 and January 2010). After fixing in formalin the proximal epiphyses were marked with India ink. Femurs were placed whole in cassettes, paraffin embedded, sectioned longitudinally at 4 μm, and stained with hematoxylin and eosin. The diaphyseal cortical thickness was measured in 3 areas—proximal, midbody, and distal, if possible.

A Mann-Whitney *U*-test and an extended Mantel-Haenszel test was used to compare the midrange bone measurements of femurs at the middiaphyseal point between wild pitfall trap and captive frogs.

Analysis of risk factors for MBD and osteofluorosis and response to treatment

Husbandry: Each institution through 2008 had similar conditions for their indoor enclosures. Hamilton Zoo, which housed frogs in four outdoor covered and screened areas, was the exception. Frogs inside were held either individually (Otago) or in groups in temperature, humidity, and moisture-controlled environments. Details are outlined in a 2008 report to the New Zealand Department of Conservation.[24] From 2008, most institutions made substantial husbandry changes. A revised and updated summary of ambient temperature, humidity, water, lighting, diet, and supplementation for captive *L. archeyi* and *L. hochstetteri* is given (Table 1). Details are outlined in the following subsections.

Diet and nutritional analyses: All captive institutions fed frogs a variety of commonly used commercial invertebrates with only Auckland Zoo consistently using vitamin and mineral supplements.[24] From 2005 through 2008, Auckland Zoo used Miner-All Outdoor calcium/mineral supplement (Sticky Tongue Farms, Sun City, California 92586, USA) dusted onto the invertebrates directly before feeding out twice a week, and Herptivite multivitamin (Rep-Cal Research Labs, Los Gatos, California 95031, USA) dusted once a month. In February 2008, in response to the diagnosis of MBD, a calcium treatment regime was instigated.[36] Frogs were given 1–2 hr shallow baths in 2.5% calcium gluconate (Phebra Pty. Ltd., Lane Cove, New South Wales 2066, Australia) daily. Baths were discontinued after 3 wk because of an erythematous skin reaction. Along with the baths, calcitriol, a biologically active form of vitamin D (Rocatrol 0.25 μg capsules, Roche Products Ltd., Dee Why, New South Wales 2099, Australia) was given (0.2 mg/kg percutaneous s.i.d.[37]) for 2 wk.

From April 2008, several diet and supplement changes were made based on regimes used to treat a frog thought to be similar in its ecology,[38] and included the discontinuation of houseflies, and the feeding of wax moth larvae monthly instead of weekly. Another change was the dusting supplements were changed to Reptical (Aristopet, Masterpet Australia, Prestons, Sydney, New South Wales 2170, Australia) once a week and a 50:50 mixture of Reptical and Reptivite (Aristopet, Masterpet Australia) once a week. A third change was that commercial crickets were only given carrots and gut loaded with a combination of calcium carbonate powder and chick starter (ChickStarter, NRM New Zealand Ltd., Newmarket, Auckland 1145, New Zealand) for 48 hours prior to feeding, and a final change that percutaneous administration of calcium/vitamin D_3 drops (Calcivet; Vetafarm, Wagga Wagga, New South Wales 2650, Australia) once a week was started.

In August 2009, the calcium/D_3 drops were discontinued to avoid disruption to the frogs during the breeding season. In January 2010, isopods were added to the weekly diet. In June 2010, dusting supplements were changed again to straight calcium carbonate powder once a week and a 50:50 mixture of calcium carbonate and Reptivite (Aristopet; Masterpet Australia) once a week and amphipods were added to the diet.

The University of Otago started percutaneous supplementation with AviCal calcium/vitamin D_3

Table 1. Summary of husbandry conditions for *Leiopelma archeyi* and *Leiopelma hochstetteri* (adapted from Shaw and Holzapfel[24]).

	Canterbury University (2000–2005)	Auckland Zoo (2005–2011)	Hamilton Zoo (2005–2011)	University of Otago (2005–2011)
Air temperature (°C), *L. archeyi*	Controlled; 11–15°C	2005–2009: controlled; 11–15°C	2005–2008: ambient	Controlled; 12–16°C
Water temperature (°C), *L. hochstetteri*		2010: ambient	2008: water chilled 4–15°C	
Humidity (%)	Unknown	2005–2009: controlled; >85 2010: ambient	Ambient	controlled; >85
Water filtration	Ceramic filtered town supply water	2005–2007: reverse osmosis 2007–2009: charcoal and particle filtered town supply 2009: reverse osmosis	Carbon and sediment filtered town supply	2005–2009: charcoal-filtered town supply 2009: reconstituted soft water using purified MilliQ® water
Lighting	Fluorescent; seasonal day length cycle	2005–2008: incandescent; seasonal day length cycle 2008–2009: artificial UVB lighting seasonal day length 2010: mesh filtered direct sunlight	Mesh-filtered direct sunlight	2006–2009: fluorescent, 11 hr on 2009: monthly artificial UVB exposure to half of the frogs
Diet	Crickets, fruit flies, houseflies, wax moth larvae	2005–2008: crickets, fruit flies, houseflies, wax moth larvae 2008–2009: discontinued houseflies 2010: isopods and amphipods added	Crickets, fruit flies, houseflies, wax moth larvae	Crickets, locusts, houseflies, wax moth larvae
Supplements	Nil	2005–2008: Mineral-outdoor® supplement and Herptivite® dusting 2008–2009: Reptical® and Reptivite® dusting; gut loading crickets; weekly percutaneous Ca/D_3 2010: calcium carbonate and Reptivite® dusting; gut-loaded crickets	2008: weekly percutaneous calcium/D_3 to select frogs	2008: once per 2 wk percutaneous calcium/D_3 to select frogs

drops (Aristopet; Masterpet Australia) once every 2 wk in May 2008.

Hamilton Zoo started percutaneous administration of calcium/vitamin D_3 drops to frogs with obvious fractures (Calcivet; Vetafarm) weekly in October 2008.

The Auckland Zoo diet (including added supplements) was analyzed twice by New Zealand Laboratory Services Ltd. (Penrose, Auckland 1642, New Zealand). Calcium, phosphorus, vitamin A, and vitamin D_3 levels were measured in April 2008 (before any changes). In December 2008, the new diet was submitted for analysis of calcium and phosphorus only. In July and August 2009, the diet with all supplements was analyzed with computer software Zootrition (V2.6; St. Louis, Missouri 63128, USA). Zootrition estimated nutrient levels based on published values of similar invertebrates and inputted values of the supplements.

Water purification and analyses: Coromandel: In December 2008, a 1,000-ml water sample was analyzed (Hill Laboratories, Hamilton 3240, New Zealand) from Tapu Stream located in frog habitat in the Coromandel region (–36.59/175.34).

Auckland Zoo: From 2005 to 2007, the Auckland Zoo used reverse-osmosis water filtration to purify town water supplied to the native frog house to moisten the soil substrate. In February 2007, this was changed to a charcoal and particle filter (Taylor Purification, Mt. Wellington, Auckland 1060, New Zealand). In November 2008, a 1,000-ml water sample water was analyzed (WaterCare Laboratory Services, Airport Oaks, Auckland 2022, New Zealand) for a variety of metals and minerals. In February 2009, the water was changed back to reverse osmosis and reanalyzed.

University of Otago: The University of Otago used charcoal filters (MATRIKX +CTO®/2 32-250-125-975, KX Technologies, West Haven, Connecticut 06516, USA) to purify the town water supply and misted this daily onto paper towels in the frog enclosures. A 1,000-ml water sample was analyzed (Citilab Analysis Consultants, Dunedin 9054, New Zealand) in February 2009. In March 2009, the water was changed to reconstituted soft water[37] made up with purified Milli-Q water (Millipore, Billerica, Massachusetts 01821, USA).

Hamilton Zoo: The Hamilton Zoo native frog enclosures used sediment and charcoal filtered (Matrikx CR1, KX Technologies) town water supply for their semiaquatic exhibits. In December 2008, a 1,000-ml water sample was analyzed (Hill Laboratories, Hamilton 3240, New Zealand). In June and November 2008 all enclosures had chillers installed to cool the water supply (Hailea Model HC130A, Hailea Industrial Zone, RaoPing County, Guangdong 515700, China).

Canterbury University: At Canterbury University ceramic filtered town water (model and manufacturer unknown) was supplied to frog enclosures from 2000 to 2005. Published water analyses were obtained from the Christchurch City Council (Christchurch 8140, New Zealand).

UVB light protocols and measurements: From 2000 to 2009, none of the captive institutions with indoor facilities provided UVB light.

Whareorino Forest: In October 2008, Auckland Zoo and Department of Conservation field staff took UVB readings with a digital ultraviolet radiometer (Model ST-6, range 0–1999 $\mu W/cm^2$; Zoo Med Laboratories, Inc., San Luis Obispo, California 93401, USA) over 7 days in frog habitat in the Whareorino forest of the Waikato region (–38.42, 174.68). Readings were taken in the early morning, as that is when *L. archeyi* bask (B. Bell, pers. comm.).

Auckland Zoo: In response to these findings, in February 2009 Auckland Zoo outfitted each indoor enclosure with Repti-Glo 2% bulbs (Exoterra; Rolf C. Hagen Corp., Mansfield, Massachusetts 02048, USA) 20 cm above the substrate. Lights were on seasonal day-length timers and shaded shelters were provided. Readings with the same ultraviolet radiometer were taken at substrate level and compared with readings from 5% bulbs. In July 2010 the frogs were moved to outdoor meshed enclosures with filtered sunlight. UVB readings at substrate level were taken weekly in the early morning.

University of Otago: In 2009, The University of Otago instigated a UVB regime based on experience at Durrell Wildlife Conservation Trust. Six frogs received UVB radiation of 300 $\mu W/cm^2$, once per month, for 20 min. Frogs were placed on wet paper towels in a transparent plastic box with a perforated floor and walls covered by a perspex transparent sheet. A UVB light (OSRAM, model E27/ES, Ultra-Vitalux; Impel New Zealand, 917 Onehunga, Auckland 1066, New Zealand) was placed approximately 50 cm above the substrate. The UVB radiance was measured with a UV radiometer (DSE-100X; Spectroline, 956 Brush Hollow Road, Westbury, New York 11590, USA) and with its corresponding sensor (DIX 300A, Spectroline).

Hamilton Zoo: At Hamilton Zoo, UVB light was measured during 1 day in 2010 (enclosures had not been altered). Readings were taken inside each of the enclosures at frog level in a variety of locations every hour from 8 AM to 5 PM (E. Shaw, unpubl. data). UVB readings were measured with the use of a Solarmeter Digital Ultraviolet Radiometer (Model 6.2, range 0–1999 μW/ cm^2; Solartech Inc., Harrison Twp,, Michigan 48045, USA).

Investigating association of fluoride with mortality rates

Mortality rates were calculated for each year for the Auckland Zoo and the Hamilton Zoo collection. They were not calculated for the University of Otago, as only one frog died, which was euthanized. The numerator was the number of frogs that died that year. The denominator was the number of frogs at the beginning of the year + number of frogs at end of year all divided by 2.

Mortality rate trends were calculated for Auckland Zoo only with the same method, for 2005–2007 (before fluoride was introduced), 2007–2009 (during fluoride exposure and 10-mo fluoride withdrawal) and 2010–2011 (fluoride removed for 2–3 yr) and compared with the use of a two-tailed Fisher's *P* test.

Statistical analyses

Winpepi Version 11.4 (http://www.brixtonhealth.com/pepi4windows.html) was used for statistical analyses.

RESULTS

Extent of signs of MBD

Retrospective review of postmortem reports: Examination of postmortem reports from 2005 through 2006 from Massey University revealed one fracture in 20 *L. hochstetteri* and none in 6 *L. archeyi* from Canterbury University, and one maxillary abnormality in 18 *L. archeyi* from Auckland Zoo.

Skeletal radiography: Radiographs from the free-living frogs that died or were collected in pitfall traps (29 *L. archeyi* and 12 *L. hochstetteri*) revealed no abnormalities. Their femurs and humeri had radiolucent epiphyses, a natural diaphyseal curve, radiopaque cortices, and radiolucent medullary cavities (except at the middiaphysis, which was radiopaque). The phalanges were not prominent (Fig. 1A). Frogs <13 mm SVL appeared radiolucent.

Radiographs of *L. archeyi* from the Auckland Zoo in February 2008 revealed 22 of 62 frogs (35%) had complete or folding fractures in one or more sites (radioulna, femur, tibiafibula, calcaneum, urostyle, and sacrum). In April 2008, no new fractures were found from 58 frogs radiographed. In August 2008, 1 of 17 frogs radiographed had new folding and complete femoral fractures. In March 2009, one of nine frogs radiographed had a new radioulnar fracture. In March 2010, 12 of the remaining 26 frogs (46%) had fractures. Most frogs appeared to have normal density when compared to the free-living frogs of the same SVL, but there were some that appeared to have radiolucent cortices of the long bones. Frogs that did not have any fractures had femurs and humeri with a natural diaphyseal curve. The spacing of the metacarpals, metatarsals, and phalanges was prominent. The proximal and distal ends of the diaphysis of the long bones appeared denser than free-living frogs (Fig. 1B–E).

Radiographs of *L. archeyi* from the University of Otago in October 2008 revealed 8 of 12 frogs (67%) had complete or folding femoral fractures.

Radiographs of *L. hochstetteri* from the Hamilton Zoo in October 2008 showed 9 of the 24 frogs (38%) had complete or folding femoral and spinal fractures.

Radiographs from deceased frogs at Canterbury University (that had not been necropsied or had a postmortem exam), revealed 1 *L. archeyi* of 31 frogs (3%; 23 *L. archeyi* and 8 *L. hochstetteri*) was fractured. No other abnormalities were detected.

The rate of fractured frogs was statistically greater when comparing each institution individually and, when combining all institutions, to Canterbury University ($P = 0.00$; odds ratio = 19.8 [95% confidence index {CI} 3–829]).

Diagnosis of osteofluorosis

Microcomputer tomography scans of femurs: All parameters were significantly different between the femurs from the pitfall trap frogs and the captive Auckland Zoo frogs at the middiaphyseal point only (Fig. 2). The captive femurs were significantly greater in bone volume ($P = 0.03$), bone surface ($P = 0.03$), cross-sectional thickness ($P = 0.03$), and mean total cross sectional bone perimeter ($P = 0.05$). Grossly, the micro-CT scans showed the captive frog femurs were misshapen (Fig. 3).

Histology: Femurs from adult free-living *L. archeyi* generally had round proximal and distal epiphyses composed of well-organized cartilage surrounded by a thin layer of connective tissue.

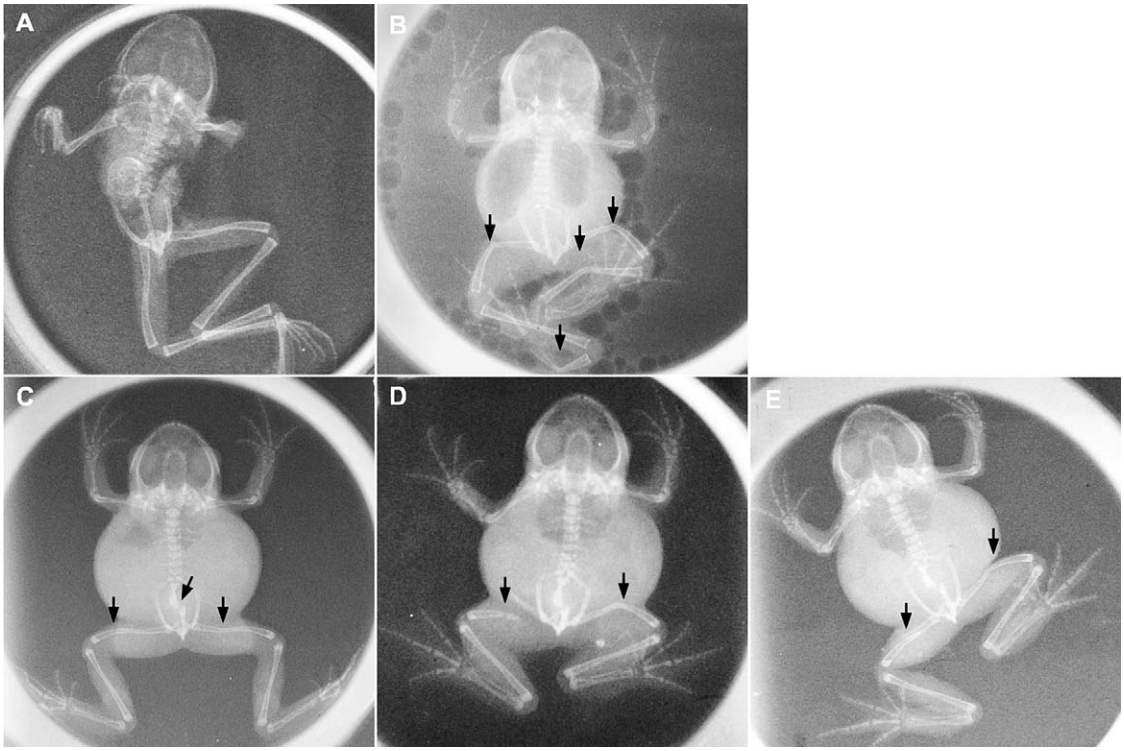

Figure 1. Radiographs of *Leiopelma archeyi*. (**A**) Normal adult wild frog. Note the natural diaphyseal curve of the femurs. (**B**) Radiograph of a captive frog showing multiple long bone fractures (arrows). (**C–E**) Radiographs of a different captive frog over time. Arrows indicate folding and complete fractures. Note dense proximal and distal diaphysis when compared with the wild frog, which may indicate increased trabeculae. (**C**) February 2008, note fractures of urostyle and right femur. (**D**) August 2008 (7 mo post calcium therapy), note complete fracture of left femur. (**E**) March 2009 (1 mo post UVB light addition and removal of fluoride), left femoral fracture is in better alignment.

The proximal diaphysis was filled with a cartilaginous marrow, and the cortices contain mineralized bone. Most of the diaphysis has well-mineralized bone with an adipose filled marrow. Closer to the distal epiphysis the marrow is again filled with cartilage. None had any trabecular bone nor were any fibroblasts, osteoblasts, or osteoclasts seen (Fig. 4A–C).

Nine of the 12 femurs from captive *L. archeyi* had misshapen proximal and/or distal epiphyses, which were also composed of well-organized cartilage, and angular deformities on one side of the diaphysis (Fig. 4D, E). There were increased fibroblasts and osteoblasts in the areas of bone remodeling. When present, new cartilage in calluses was slightly irregular in organization (Fig. 4F). Seven of the 12 had trabecular bone just distal to the proximal epiphysis or proximal to the distal epiphysis (Fig. 4G). The diaphysis was often poorly mineralized and had both endosteal new bone and periosteal bone (hyperostosis) that were also poorly mineralized. The bone marrow contained mainly adipose tissue (Fig. 4H–L).

The midrange cortical diaphysis thickness measurements were not statistically different between the pitfall trap frogs and the captive frogs.

The histology on other organs did not reveal any primary causes of death. Many frogs had evidence of hepatocelluar steatosis and fat body steatitis. No renal lesions were seen.

Analysis of risk factors for MBD and osteofluorosis and response to treatment.

Husbandry: Nutritional analyses: Comparisons of the main nutrients analyzed (calcium, phosphorus, vitamin D_3, and vitamin A) are presented in Table 2. The calcium:phosphorus (Ca:P) ratio was 4.4:1 for the original Auckland Zoo diet and supplements and this only differed slightly (4.9:1) from the new diet from April 2008. These values include all supplements fed to and dusted on the invertebrate just before testing.

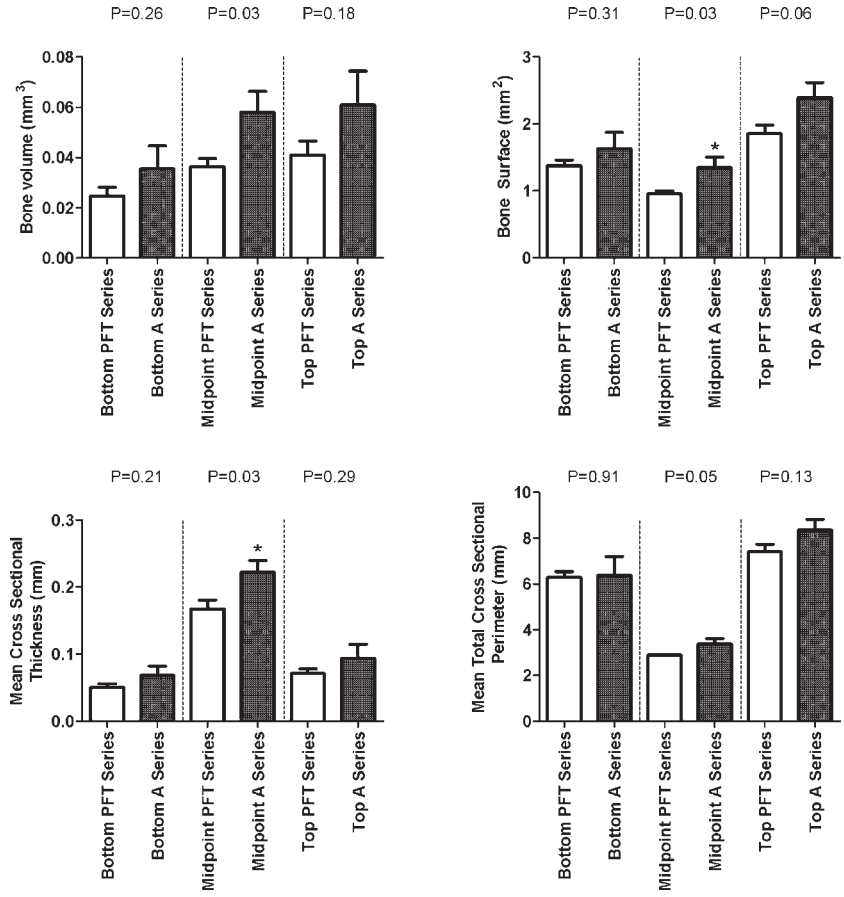

Figure 2. Micro-CT results. The graphs compare measurements on femurs from frogs in two groups (PFT = wild pitfall trap *Leiopelma archeyi* and A = captive Auckland Zoo *Leiopelma archeyi*) in selected parameters. Parameters were statistically different between the pitfall trap femurs and the captive Auckland Zoo femurs at the midpoint only. The captive femurs were significantly greater in bone volume ($P = 0.03$), bone surface ($P = 0.03$), cross-sectional thickness ($P = 0.03$) and mean total cross sectional bone perimeter ($P = 0.05$).

The Ca:P values calculated by Zootrition were higher (Table 3). The Ca:P ratio was increased from 10:1 for the old diet to 11:1 for the new diet when calcium carbonate was added to the supplements. The Ca:P ratio of the invertebrate portion of the diet alone was 0.13:1 but these values were based on crickets that were not gut-loaded, so are likely to be underestimations.[10] Removing the percutaneous calcium/vitamin D_3 supplement did not change the calcium or D_3 values.

Water analyses: Comparisons of various minerals and metals (fluoride, dissolved calcium, total copper, total iron, and total lead) in the water from different institutions found copper, iron, and lead were found in negligible amounts in all water sources (Table 4). Fluoride was present in water from Auckland Zoo's particle-filtered water, which had the highest levels, and particle-filtered water from Hamilton Zoo and Otago University, but only in negligible amounts in the Canterbury University filtered town water, Coromandel frog habitat stream water, and Auckland Zoo reverse-osmosis water. Calcium was the highest in the University of Otago town supply water and only in negligible amounts in the Auckland Zoo reverse-osmosis water. The pH was not tested in all water samples.

UVB light: The UVB readings in Whareorino Forest were 1–2 μW/cm².

At Auckland Zoo in the indoor frog enclosures, Exoterra 2% and 5% bulbs gave readings of 1–2 μW/cm² and 15–16 μW/cm² respectively at substrate level. In the outdoor enclosures the readings of natural light were 1–4 μW/cm² at the substrate level.

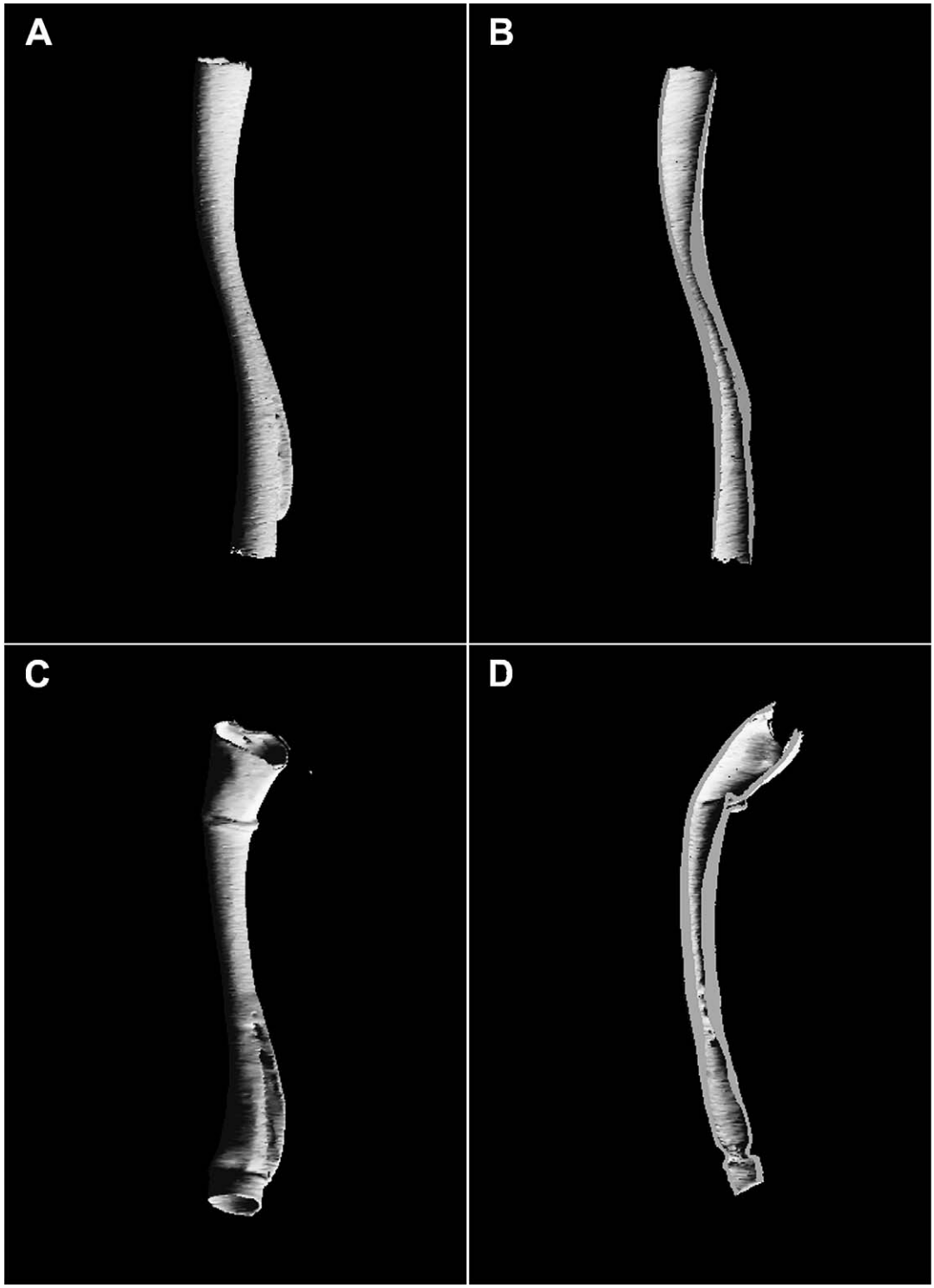

Figure 3. Micro-CT images of femurs from adult *Leiopelma archeyi*. (**A**, **B**): Normal wild frog. (**C**, **D**): Captive frog. Note the folding fracture.

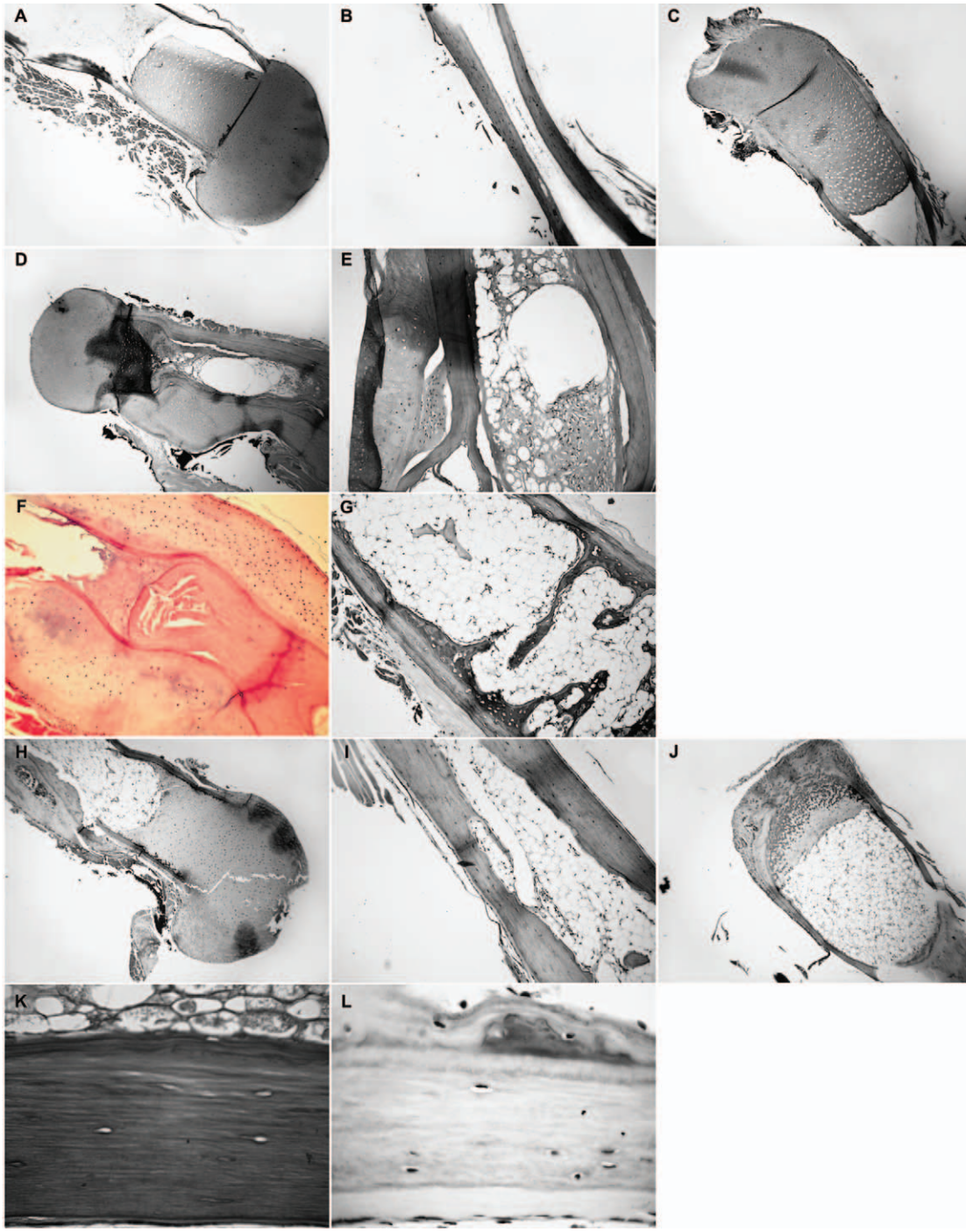

Figure 4. Histologic sections of femurs of *Leiopelma archeyi* hematoxylin and eosin stain. (**A**–**C**) Normal femur (proximal epiphysis, diaphysis, distal epiphysis) from adult wild frog (×40). (**D**, **E**) Sections from a captive Auckland Zoo frog showing a misshapen proximal epiphysis, angular diaphyseal deformity, and hyperostosis (×40, ×100). (**F**) Captive frog with a callus at a previous fracture site middiaphysis. The cartilage is slightly irregular in organization (×100). (**G**) Captive frog with trabeculae proximal diaphysis (×100). (**H**–**J**): Captive frog. The proximal and distal epiphyses are misshapen; the diaphysis is poorly mineralized and has mild hyperostosis (×40, ×100, ×40). (**K**) Wild frog; normal cortex (×400). (**L**) Captive frog; one side of cortex with both endosteal new bone and periosteal bone, poorly mineralized (×400).

Table 2. Summary of nutritional analyses of native frog diets at Auckland Zoo, including all supplements.

	Calcium (mg/100 g)	Phosphorus (mg/100 g)	Ca:P ratio	Vitamin A (μg/100 g)	Vitamin D_3 (μg/100 g)
April 2008	1,200	270	4.4:1	1.3	<0.1
December 2008	3,900	790	4.9:1	Not done	Not done

At the University of Otago, UVB readings were 10 μW/cm² at the substrate level.

At Hamilton Zoo, the average maximum readings ranged from 0.3 to 1.9 μW/cm². The absolute maximum UVB readings for 1 day were 4–24 μW/cm² (E. Shaw, unpubl. data).

Investigating association of fluoride with mortality rates

Mortality rates for Auckland Zoo were 2005 (9.4%), 2006 (17.9%), 2007 (6.2%), 2008 (31.2%), 2009 (52.1%), 2010 (7.8%), and 2011 (4.1%).

The combined mortality rate trend from 2007–2009 (fluoride exposure) was significantly greater than the combined mortality rate trend for 2005–2006 ($P = .00$; odds ratio = 15.4 [95% CI 5–47]) and 2010–2011 (fluoride absent) ($P = .000$; odds ratio = 43.8 [95% CI 9–266]).

Mortality rates for Hamilton Zoo were 2006 (14.3%), 2007 (5.3%), 2008 (34.9%), 2009 (12.2%), 2010 (4.0%), and 2011 (9.5%). The year 2008 had significantly higher mortality than all years ($P < 0.05$) except 2009.

DISCUSSION

Extent of signs of MBD

The retrospective radiographs, post-mortem findings and clinical reports of hunched posture and tetanic spasms supported the conclusion that signs of MBD had been present in both the Canterbury University and Auckland Zoo collections for some time, but at a lower incidence than the index cases at Auckland Zoo in 2007.

Diagnosis of MBD and osteofluorosis

The abnormal bone structure observed with the use of micro-CT, and histology led to the diagnosis of MBD and osteofluorosis and was supported by analysis of risk factors discussed below. In typical MBD without the complication of fluorosis, the bones of the captive frogs would have had significantly thinner cortices and less bone volume than in wild frogs.[38] However, micro-CT showed that the captive *L. archeyi* had significantly increased cross-sectional bone thickness and bone volume, consistent with fluorosis. Fluoride is a cumulative element that increases metabolic turnover of bone.[29] Depending on the levels of fluoride, both preformed and new bone can be altered with impaired mechanical properties.[7,32] Compared to other bone anabolic factors, fluoride remains the most potent agent inducing uncoupling between bone resorption and bone formation in favour of formation, thus resulting in an increased bone volume, but not increased strength.[7]

Histology showed bones were abnormal: skeletal fractures, hyperplasia, periosteal growth (hyperostosis), and thickening of trabeculae. In mammals, fluorosis can cause skeletal fractures, hyperplastic bones, cortical osteoporosis, osteopenia, periosteal growth (hyperostosis), thickening of trabeculae, exostoses, osteopetrosis, hypertrophic joints, and diaphyseal widening.[8,16] Histology of the femurs of the frogs supported the diagnosis of fluorosis, but was not as sensitive as micro-CT in measuring microparameters of the bone. Metabolic bone diseases can look different

Table 3. Composition of selected nutrients with the use of Zootrition analyses for 0.31 g dry matter of Auckland Zoo native frog diet.

	Calcium (%)	Phosphorus (%)	Ca:P ratio	Vitamin A (IU/g)	Vitamin D_3 (IU/g)	Fat (%)	Protein (%)	Magnesium (%)
July 2009 with all supplements	4.94	0.44	11:1	195.25	12.69	2.98	16.6	0.04
July 2009 with only percutaneous supplements	4.94	0.44	11:1	195.25	12.69	2.98	16.6	0.04
July 2009 without supplements	0.06	0.44	0.13:1	0	0	2.98	16.6	0.04
August 2009 with supplements	6.60	0.68	10:1	175.79	4.39	20.69	42.07	0.08
August 2009 without supplements	0.08	0.67	0.12:1	0	0	20.69	42.07	0.08

Table 4. Comparisons of various components in different water sources.

Institution and type of filtration	Fluoride (mg/L)	Chloride (mg/L)	Dissolved Calcium (mg/L)	Dissolved Magnesium (mg/L)	Total Copper (mg/L)	Total Iron (mg/L)	Total Lead (mg/L)	pH
Auckland Zoo reverse osmosis	0.06	0.39	0.02	not tested	0.013	<0.002	0.0033	not tested
Auckland Zoo charcoal and particle filter	0.94	13.9	9.8	not tested	0.001	<0.002	<0.0001	not tested
Hamilton Zoo carbon filter	0.25	19	9.6	2.2	0.007	0.10	0.00053	7.1
University of Otago Dunedin town supply	0.85	14	22.5	not available	<0.03	<0.03	not available	not available
Canterbury University Christchurch unfiltered town supply*	<0 .01	5.0	12.0	1.5	not available	not available	not available	not available
Tapu Stream in Coromandel native frog area	<0.05	14	5.3	2.4	<0.001	0.1	<0.0001	7.8

[a] Data taken from published city water analysis in 2010; actual water used passed through ceramic filter some values may be lower.

histologically in each species and sometimes in different bones, and often etiologies will vary in appearance.[32] Relying solely on histology can create confusion diagnostically.[32]

Fluorosis in mammals and fish can be diagnosed by proof of exposure along with bone fluoride levels.[8,16,26] In the case of *L. archeyi* at Auckland Zoo, testing bone fluoride levels had logistical prohibitions, but the micro-CT and histology evidence along with proof of fluoride exposure (discussed below) strongly suggested the diagnosis of osteofluorosis. However, as histology and micro-CT were not done with the frogs at the University of Otago and at Hamilton Zoo, the evidence is not as strong and the diagnosis has been extrapolated from the Auckland Zoo situation and the literature. This extrapolation is difficult, as the level of fluoride to cause toxicity in these species and amphibians in general is unknown, and there are many factors that can alter what level of fluoride will cause toxicity. Reported toxic levels in fish were compared to levels of exposure in *Leiopelma* as the mode of exposure is similar. Freshwater chinook salmon (*Oncorhynchus tshawytscha*) and coho salmon (*Oncorhynchus kisutch*) are reported to show clinical signs of fluorosis at 0.20 mg/L, whereas rainbow trout (*Oncorhynchus mykiss*) in the Firehole River in Yellowstone National Park (Montana, USA) are healthy with fluoride levels up to 14 mg/L.[5] Analysis of the water supplies in 2008 showed all institutions used water that contained a greater fluoride content (0.25–0.94 mg/L) than the frogs would be exposed to in their natural habitat, as both stream water and typical rainwater[18] are normally fluoride free. Canterbury University did not have fluoride in the water, and Auckland Zoo had fluoride-free water until the reverse-osmosis water purification filtration was replaced in early 2007 with charcoal filtration.

Risk factors for MBD and osteofluorosis

The increase in water fluoride levels preceding the outbreak of pathology consistent with osteofluorosis suggested that this was the cause. Further analyses of long-standing husbandry conditions showed likely causes of underlying MBD were hypocalcemia and the lack of UVB exposure when compared with wild conditions.

Dietary imbalances are considered the most common cause[36] of osteodystrophies, and a known factor in fluorosis. In hypocalcemic mammals, the toxic effects of fluoride can manifest at even marginally high exposure and exaggerate the metabolic effects of calcium deficiency on

bone.[13,29,30,32] In aquatic organisms, there is evidence that calcium levels in the water can affect the degree and clinical appearance of fluorosis seen.[5] Therefore, when compared to sensitive fish species, the levels of fluoride in the water that the frogs were exposed to could have been in the toxic range, but the nutritional status of the frogs and/or the amount of calcium in the water would likely have affected the rate and degree of affected frogs. However, toxicity could be even more complex as other factors can affect the susceptibility of fish to fluoride such as species, an individual's size, water temperature, and the mineral content of the water (e.g., chlorine).[5] Certainly, if using fluoridated water could not be avoided then all these factors would need to be carefully analyzed and considered to avoid toxicity. In most cases, it would be more efficient and cheaper to filter out the fluoride.

In this current study, the nutritional analyses to assess changes to the diet were flawed. With the use of the laboratory data and Zootrition analyses, it appeared that the Auckland Zoo diet had a very high Ca:P ratio (5–9:1). However, this may not reflect the frog's true diet, as it has been shown that dusted supplements are removed by the invertebrates within an hour.[15] At Auckland Zoo, frogs are fed twice a week during daylight hours. As *Leiopelma* are nocturnal, and rarely observed eating during the day, it is likely that only the gut-loaded crickets and percutaneous supplements were contributing to the calcium and vitamin D_3. Because the commercial invertebrates without gut-loading or dusting contained an insufficient Ca:P ratio (0.13:1) it was decided to change the diet to resemble that of wild *L. archeyi* to avoid relying on supplementation solely to address the calcium deficiency. Stomach-contents analyses have shown that wild *Leiopelma* eat a high proportion of terrestrial crustaceans and other invertebrates (woodlice, snails, millipedes, silverfish) with a much higher Ca:P ratio than most commercial invertebrates.[20,25] Further analyses should be done to evaluate the current diet with the new invertebrates and gut-loaded crickets only.

Comparison of exposure to UVB light in captive and natural habitats indicated a lack of UVB exposure in the indoor enclosures was likely another contributing factor to the underlying MBD. Originally UVB lights were not recommended by the Native Frog Recovery Group (NFRG) as *L. archeyi* was thought to be completely nocturnal. In 2009 it was noted by an established *Leiopelma* field researcher that *L. archeyi* did bask in early-morning dappled light (B. Bell, pers. comm.). With this new evidence, UVB lights were added to the indoor enclosures at Auckland Zoo and the University of Otago. Two different regimes were used and adverse effects were not seen at either institution. An additional benefit at Auckland Zoo was a marked increase in plant growth and cover, which gave the frogs potentially more places to hide and climb.

Response to treatment

Since eliminating fluoride, the addition of UVB light, and increasing the inherent calcium of the captive diet, there have been no new fractures, tetanic spasms (S. Shaw, unpubl. data) have been eliminated, and mortality at Auckland Zoo is significantly decreased.

At the University of Otago, there was a similar reduction in fractures since eliminating fluoride, adding UVB, and adding calcium supplements.

Although water at Hamilton Zoo still contains fluoride, repeat radiographs of frogs treated with percutaneous calcium show callous formation and new fractures have not occurred (M. Goold, pers. comm.). As these frogs are kept outdoors with natural UVB exposure, inconsistent dietary calcium due to intermittent influx of invertebrates and variable water temperature, it is likely that the combination of factors at present is not causing fluoride toxicity in the adults. However, low reproduction and tadpole deaths (K. Goddard, unpubl. data) indicate husbandry is not optimal and fluoride could be a factor. More investigation is required to understand clearly the association of fluoride with morbidity and variable mortality at this institution if it is to remain in the water supply.

Monitoring the frog's recovery and response to treatment was also explored. Micro-CT allowed the microarchitecture of the bone to be examined and provided a true 3D model of each bone. It was an excellent tool to help diagnose the cause of MBD and would be useful to monitor the frogs' recovery. The downside is that it requires special equipment, can only be used postmortem, and requires removal of the bone.

Radiographs were very useful to visualize long bone fractures and to diagnose MBD in the collections. Although some femoral fractures were grossly visible, many were not and may have gone undetected even though a high percentage of femurs were affected (35–67%), as has been reported in captive mountain chicken frogs (*Leptodactylus fallus*).[14] However, the serial X rays of frogs under treatment taken every 3 mo over 2 yr

at the Auckland Zoo were not sensitive enough to show facial fractures or clear short-term changes in bone density. The serial X rays at the University of Otago and Hamilton Zoo showed similar results. It has been reported that bone density needs to be altered by 30–50% before changes can be seen radiographically,[12,32] thus making it difficult to use as a response to treatment monitoring tool if bone density changes are under this level. Further investigation into imaging techniques for small bones in live frogs is needed. One possibility may be dual-energy X-ray absorptiometry (DEXA), which has been used for measuring bone mineral density in live green iguanas and humans.[34,39] Another is phase contrast X-ray imaging, which was used to monitor treatment of MBD antemortem in the great barred frog (*M. fasciolatus*).[38]

It is unknown how long fluoride could remain in the bones of amphibians and if it will continue to affect the health, and possibly future reproduction of these frogs if it is present. In humans, there is evidence of clearing in 1.5–8 yr.[30,34] and in bongo antelope (*Tragelaphus eurycerus isaaci*), 18 months.[16]

Metabolic bone disease, regardless of the cause, can be devastating to an amphibian collection and reduce the success of conservation programs relying on captive breeding. However, it is an entirely preventable disease, if knowledge of the normal water conditions, natural diet, and UV exposure of particular species can be obtained and if there are resources to implement husbandry changes.

Management recommendations

A priority should be to investigate optimal husbandry before frogs are brought into captivity. Animal-care staff should be trained to recognize early clinical signs of metabolic bone disease. Finally we recommend that the following factors are investigated for amphibians in captivity to decrease the prevalence of this disease. Factors include monitoring water quality by periodic water analyses measuring a wide range of minerals such as calcium, chloride, fluoride, magnesium, copper, iron, and lead; and comparing values to that of the natural habitat of the amphibian. In addition, the use filters that remove heavy metals and fluoride as a minimum is recommended. Moreover, another factor is the diet, and conducting nutritional analyses of the captive diet and investigating the natural diet of the species is recommended. The goal is to feed the captive amphibians a diet of similar nutritional composition as the wild diet. If possible, the use of some invertebrates with an inherently high Ca:P ratio (such as terrestrial crustaceans)[20] and reducing reliance on dusted supplements is recommended. The goal for diets is for a Ca:P ratio of 1.5:1.0.[35] Finally, monitoring UVB light and providing UVB light with appropriate shelter unless there is strong evidence that the species is not naturally exposed. Natural sunlight is preferred for reptiles and this may also apply to amphibians.[17]

Acknowledgments: Funding for histology was provided by the New Zealand Wildlife Society Marion Cunningham Grant. Thanks to Melanie Farrant and Nicole Czerinak, veterinary nurses from the New Zealand Centre for Conservation Medicine, for assistance in taking radiographs. Thanks to Auckland Zoo native frog keepers Natalie Clark, Andrew Nelson, and Tanya Shennan, and curator Ian Fraser, for their support in the investigation. Thanks to Kara Goddard, native frog zookeeper at Hamilton Zoo, for her support in the investigation. Thanks to Dr. Bethany Jackson and Kirsten Derry at the New Zealand Centre for Conservation Medicine for assistance in data collection. Thanks to Dr. Sam Young of Mogo Zoo and Dr. Kevin M. Wright for medical advice in treating these frogs. The authors also thank Dr. Richard Norm, Dr. Maurice Alley, and Dr. Brett Gartrell from Massey University for their assistance in histology. Thanks to the many Maori Iwi tribes for supporting native frog conservation, including: Hauraki Maori Trust Board, Mataora No. 1 & 2 Block Inc., Moehau Nga Tangata Whenua, Ngati Hei Trust, Ngati Huarere, Ngati Maru ki Hauraki Inc, Ngati Paoa Trust, Ngati Pukenga ki Waiau Society, Ngati Rahiri Tumutumu, Ngati Rongo U Charitable Trust, Ngati Tai Umupuia Te Waka Totara Trust, Ngati Tawhaki, Ngati Whanaunga, Te Kupenga O Ngati Hako Inc, Te Patukirikiri Iwi Inc,, Te Runanga O Ngati Pu, Te Ruunanga A Iwi o Ngati Tamatera. and Te Uringahu Ngati Maru Manaia. Many thanks to Lisa Daglish, Paul Gasson, Amanda Haigh and Oliver Overdyck at the New Zealand Department of Conservation for support and assistance with samples and permits.

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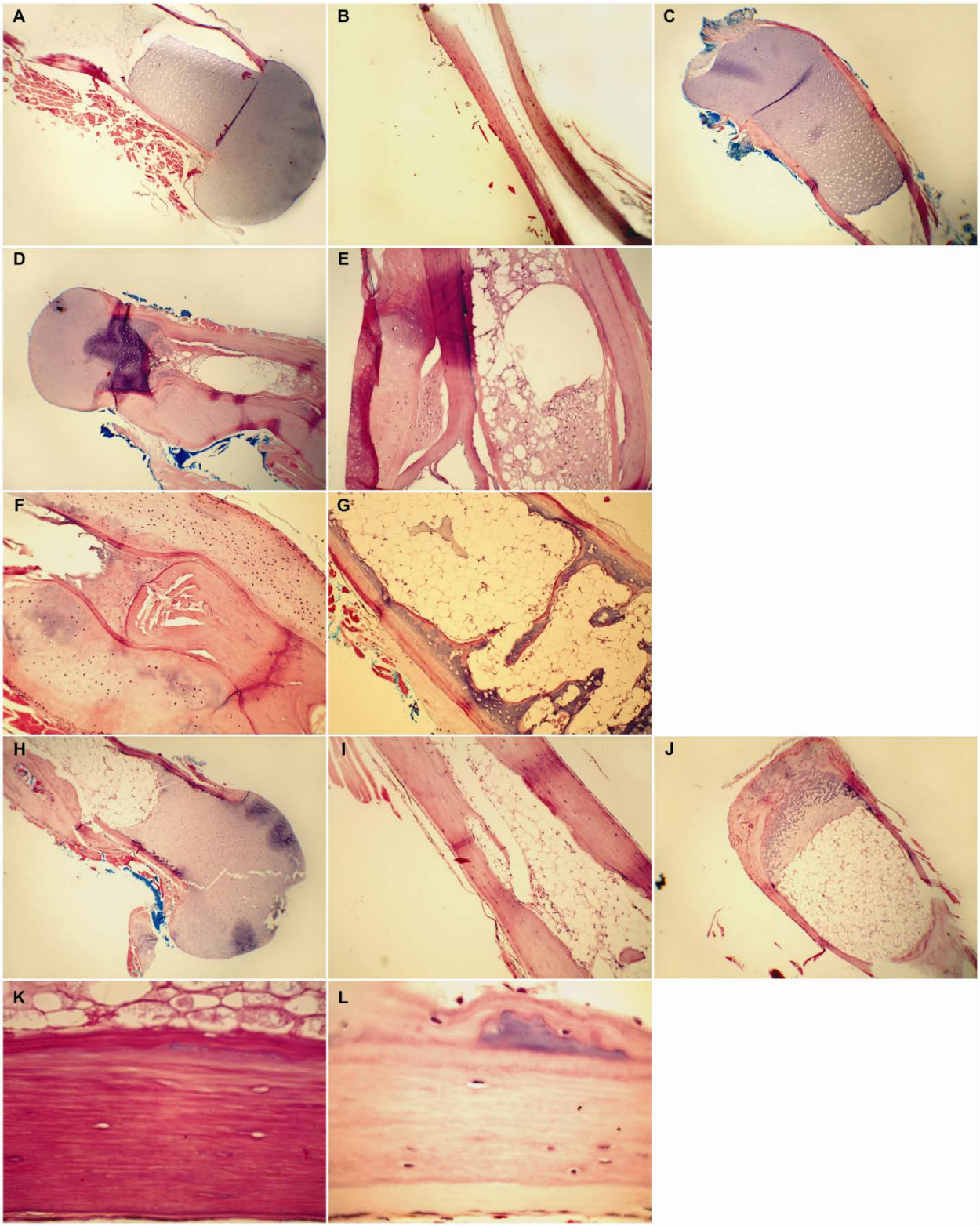
Received for publication 19 December 2011

Figure 5. Histologic sections of femurs of *L. archeyi* H and E stain in colour. **A-C:** Normal femur (proximal epiphysis, diaphysis, distal epiphysis) from adult wild frog (40x). **D-E:** Sections from a captive Auckland Zoo frog showing a misshapen proximal epiphysis, angular diaphyseal deformity and hyperostosis (40x, 100x). **F:** Captive frog with a callus at a previous fracture site mid-diaphysis. The cartilage is slightly irregular in organization (100x). **G:** Captive frog with trabeculae proximal diaphysis (100x). **H-J:** Captive frog. The proximal and distal epiphyses are misshapen, the diaphysis is poorly mineralized and has mild hyperostosis (40x, 100x, 40x). **K:** Wild frog; normal cortex (400x). **L:** Captive frog; one side of cortex with both endosteal new bone and periosteal bone which are poorly mineralized (400x).



Consent of Authors for previous published document.

	Details of publication(s) on which chapter is based	Nature and extent of the intellectual input of each author	I confirm the candidate's contribution to this paper and consent to the inclusion of the paper in this thesis	Name :	Signature
Chapter # FOUR	Shaw, S.D., P.J. Bishop, C. Harvey, L. Berger, L.F. Skerratt, K. Callon, M. Watson, J. Potter, R. Jakob-Hoff, M. Gaold, N. Kunzmann, P. West, and R. Speare. 2012.Accepted. Fibrosis as a probable factor in metabolic bone disease in captive New Zealand native frogs (Leiopelma species).Journal of Zoo and Wildlife Medicine.	Contributed all data from University of Otago ; contributed to methods and results of same dataset; editing of MS	Yes that is correct	Phillip Bishop	
	same	Contributed by helping Sr author to review and interpret all histology slides . Contributed to drafting methods, results, photos and discussion pertaining to histology section. Editing of MS	Yes that is correct	Catherine Harvey	
	same	Contributed by reviewing all cases with Sr author and assisting with drafting and all edits of MS including rebuttal.	Yes that is correct	Lee Berger	
	same	Contributed by assisting Sr author with all analyses of data and statistics. Contributed to editing methods and results of these analyses as well as assisting in organization and layout of the MS.	Yes that is correct	Lee Skerratt	
	same	Contributed by performing micro computer tomography of the femurs and the data analysis of the micro CT results. Assisted in drafting methods and results	Yes that is correct	Karen Callon	
	same	Contributed by performing micro computer tomography of the femurs and the data analysis of the micro CT results. Assisted in drafting methods and results section of micro CT.	Yes that is correct	Maureen Watson	
	same	Contributed by supervising methods done; reviewing cases with Sr author; ideological discussions and assisting with editing of the MS .	Yes that is correct	John Potter	
	same	Contributed by supervising methods done; reviewing cases with Sr author; ideological discussions and assisting with editing of the MS .	Yes that is correct	Richard Jakob-Hoff	
	same	Contributed all data from Hamilton Zoo ; contributed to methods and results of same dataset; editing of MS	Yes that is correct	Mike Gaold	
	same	Contributed by data collection of UVB measurements in the field at Whareorino and assisting in collecting data for nutritional analyses at Auckland Zoo, Assited with drafting of captive husbandry methods section for Auckland Zoo. Contributed by collection of morphological data of captive frogs and material for nutritional analyses at Auckland Zoo; contributed to general ideological discussions; assited in general editing of the methods section pertaining to Auckland Zoo.	Yes that is correct	Nicole Kunzmann	
	same	Contributed by supervising methods done; reviewing cases with Sr author; ideological discussions and assisting with editing of the MS .	Yes that is correct	Peter West	
	same		Yes that is correct	Richard Speare	

Chapter 5: Nematode and ciliate nasal infection in captive Archey's frogs (*Leiopelma archeyi*)

Preamble

Chapter Two and Chapter Four describe some of the causes for mortality in captive *Leiopelma* spp.. One of the original goals of this project was to examine dead frogs from the wild for diseases. However, I received only one wild *Leiopelma archeyi* to post-mortem and no significant pathology was found. I realized while serving as the primary veterinarian for captive *L. archeyi* at Auckland Zoo that although little was known about diseases in the wild, there was also little known about diseases and therapy in captive frogs. Therefore, it was important to report anything that could improve the clinical knowledge and management of diseases of this genus to improve the success of the captive breeding programmes.

This chapter is the original manuscript as published in a peer-reviewed journal: Shaw, S.D., Lynn, D., Yeates, G., Zhao, Z., Berger, L., Jakob-Hoff, R., (2011). Nematode and ciliate infection in captive Archey's frogs (*Leiopelma archeyi*). Journal of Zoo and Wildlife Medicine 42: 473- 479.

My contribution: 80% (detailed in co-author publication release form at the end of this chapter).

Journal of Zoo and Wildlife Medicine 42(3): 473–479, 2011

NEMATODE AND CILIATE NASAL INFECTION IN CAPTIVE ARCHEY'S FROGS (*LEIOPELMA ARCHEYI*)

Stephanie Shaw, D.V.M., M.A.C.V.S. Zoo Med., Richard Speare, B.V.Sc., Ph.D., Denis H. Lynn, B.Sc., Ph.D., Gregor Yeates, D.Sc., Zeng Zhao, B.Ag.Sci., Ph.D., Lee Berger, B.V.Sc., Ph.D., and Richard Jakob-Hoff, B.M.V.S. (Hons), M.A.C.V.S. Australasian Wildlife Med.

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Chapter # FIVE	Details of publication(s) on which chapter is based	Nature and extent of the intellectual input of each author	I confirm the candidate's contribution to this paper and consent to the inclusion of the paper in this thesis	Name :	Signature
	Shaw, S.D., R. Speare, D.H. Lynn, G.W. Yeates, Z. Zhao, L. Berger, and R. Jakob-Hoff. 2011. Nematode and ciliate nasal infection in captive Archey's frogs (Leioplema archeyi). Journal of Zoo and Wildlife Medicine 42: 473-479.	Rick Speare contributed to the MS by assisting with initial laboratory work to diagnose the nematode infection; assisted with preparation and obtaining the nematodes for identification; assisted with minor editing of the MS case reports	Yes that is correct	Rick Speare	
	same	Denis Lynn contributed to the MS by providing methods to stain the ciliate; identification of the ciliate; and contributed to writing the case report and discussion portion of the MS where it mentions the ciliate infection.	Yes that is correct	Denis Lynn	
	same	Gregory Yeates contributed to the MS by identifying the Koerneria nematode, anayzing the soil contents for nematodes, and contribution to the discussion portion of the MS where it mentions Koerneria as well as general editing of the MS and assistance with the rebuttal.	Yes that is correct	Gregory Yeates	
	same	Zeng Zhao contributed to the SM by identifaction of the Rhabditis nematode using PCR, taking pictures with measurements and providing for the MS, and contributed to writing the case report and discussion where it mentions Rhabditis.	Yes that is correct	Zeng Zhao	
	same	Lee Berger contributed to the MS by major editing and assistance with the rebuttal process.	Yes that is correct	Lee Berger	
	same	Richard Jakob-Hoff contributed by supervision of the medical portion of the case and minor editing of the MS.	Yes that is correct	Richard Jakob-Hoff	

Chapter 6: Adenomatous hyperplasia of the mucous glands in captive Archey's frogs (*Leiopelma archeyi*)

Preamble

This chapter focuses on the histopathological and epidemiological investigation of a "blistering" skin syndrome that first appeared in captive Archey's frogs at the University of Canterbury and then again at both the University of Otago and Auckland Zoo. This syndrome caused anxiety in those working with the frogs because of the striking nature of the lesions and as they affected a large number of frogs; thus raising the concern that it was a contagious disease spreading through and endangering the entire collection. These blisters appeared to be a novel disease in frogs and it was unknown if they were pathogenic. Although in Chapter Two my analyses showed that the "blistered" cohorts did not have higher mortality, it was still unknown if they were affecting the survivability of the frogs at Auckland Zoo. When Auckland Zoo had two new cases of the syndrome in frogs that had recently come from the wild, the veterinary department and the New Zealand Department of Conservation came to an agreement and permits were granted to obtain a small number of skin biopsies to allow for diagnostics including transmission electron microscopy which required fresh biopsy specimens to avoid post-mortem artefactual changes. In the end, the TEM played a role in ruling out diagnoses such as pemphigous, but due to technical difficulties, we were not able to get sufficient TEM photos of the abnormal glands. We are still trying to resection the original tissue in the hope of including any new data in the journal publication post–thesis submission. However, light microscopy was used on these fresh specimens and I was able to describe the histological and epidemiological characteristics as a new syndrome in amphibians. The aetiology was not determined, but the epidemiological analysis showed that this syndrome was not affecting the survival of the frogs, and suggested the disease was associated with a suboptimal captive environment.

This chapter is written to be submitted to the journal Veterinary Dermatology, and will be submitted post-thesis with minor changes to the content and format.

My contribution: 85%. The histopathology was sectioned and processed by Gribbles Auckland, the University of Otago or Massey University laboratories. I examined all histology slides and obtained measurements as indicated. Catherine Harvey and Maurice Alley assisted with histological descriptions. Data sheets created by the Department of Conservation were used to investigate the presence of the lesions of the frogs at the University of Canterbury. I also used Phil Bishop's notes and photographs of the lesions in the frog at the University of Otago and have verified these lesions in person. Rick Speare and Phil Bishop anaesthetized and biopsied one frog from the University of Otago. I performed all other skin biopsies in Auckland with the assistance of Rick Speare and the Auckland Zoo veterinary staff. The TEM on the Otago frog was processed by Matthew Downing at the University of Otago and reviewed by myself and Rick Speare. All the other TEM was processed by Hillary Holloway from the University of Auckland and both Rick Speare and I reviewed the sections.

Adenomatous hyperplasia of the mucous glands in captive Archey's frogs (*Leiopelma archeyi*)

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ABSTRACT: Multifocal small domed lesions occurred extensively on the ventral skin of captive adults of the endangered New Zealand native Archey's frog (*Leiopelma archeyi*). Between 2000 and 2012, lesions were found on 41% (34/83) of frogs at Auckland Zoo and 9% (1/11) at the University of Otago and lesions were not linked with an increased risk of death. The lesions had the gross and microscopic characteristics of adenomatous hyperplasia (AH) of the dermal mucous glands which are widely distributed over the skin of normal Archey's frogs. In normal frogs, mucous glands were located in the superficial dermis. The glands were circumscribed and well organized with one cuboidal to attenuated epithelial cell layer surrounding a central lumen containing mucus. Nuclei had mild anisokaryosis and were deeply basophilic with rare nucleoli. In affected frogs the size and location of lesions varied over time, even resolved completely in some animals, and sometimes reappeared. Histologically the lesions were composed of enlarged mucous glands that expanded the dermis and elevated the epidermis. They were semi-organized, with occasional acinar structures with central lumina sometimes containing mucus. Nuclei had moderate anisokaryosis and mitotic figures were uncommon. The aetiology of this adenomatous hyperplasia is unknown, but factors associated with the captive environment are the most likely cause. This is the first description of adenomatous hyperplasia of the mucous gland in amphibians.

KEY WORDS: adenomatous hyperplasia · Archey's frog · amphibian · blisters · *Leiopelma archeyi* · mucous gland

INTRODUCTION

Archey's frog (*Leiopelma archeyi*) come from an ancient lineage represented by (Bell and Wassersug, 2003) only four extant species found in New Zealand (Bell & Wassersug 2003). Weighing about 4 grams, Archey's frog is the smallest of the leiopelmatids (Shaw et al., 2012a). They are classified as endangered by the New Zealand Department of Conservation (Hitchmough et al., 2005; Newman et al., 2010) and critically endangered by international standards (IUCN, 2011). Their skin contains two types of glands: mucous and granular (Melzer et al., 2011). Although their granular glands have been well described and hypothesized to have a immune defence role against the amphibian fungal pathogen, *Batrachochytrium dendrobatidis*, the mucous glands of *L. archeyi* have only been briefly described (Melzer et al., 2011). In general, amphibian mucous glands release mucus onto the surface of the epidermis for the primary role of preventing water loss via the skin, but are also important for thermoregulation and cutaneous gas exchange (Lillywhite and Licht, 1975; Voyles et al., 2009). Amphibian skin is unique among terrestrial invertebrates in that the skin is responsible for water and electrolyte homeostasis (Shoemaker and Nagy, 1977), and disruption of these functions can cause death (Voyles et al., 2009). Hence even skin diseases in amphibians that appear mild can have severe consequences (Voyles et al., 2009). The most common skin diseases in amphibians are infectious, but various neoplasms of dermal origin have been described (Green and Harshbarger, 2001). However, there are only a few reports of neoplasms of the skin glands and none involving the mucous type of skin gland (Berger et al., 2004b; Green and Harshbarger, 2001; Speare, 1990).

Wild Archey's frogs were collected from 2000-2005 for captive breeding and research as part of the Department of Conservation Native Frog Recovery Plan (Newman, 1996). Unfortunately, the captive breeding program has been relatively unsuccessful due to various health problems such as metabolic bone disease, fluorosis, bacterial infections and skin lesions originally known as "blisters"

(Potter and Norman, 2006; Shaw and Holzapfel, 2008; Shaw et al., 2012a). These blister-like skin lesions were first noticed in the captive collection at the University of Canterbury (Potter and Norman, 2006). Investigations at that time included cytology and microbiology of a small amount of fluid aspirated from one clear "blister" in addition to transmission electron microscopy of one blister biopsy obtained post-mortem. No infectious causes were found (Potter and Norman, 2006). There is also one report of these lesions occurring in one wild *L. archeyi*, but as no histological investigation was undertaken the nature of the lesions could not be confirmed (A.Haigh, pers. comm. 2008).

The occurrence of this disease between 2000 and 2012 was investigated by collating case reports and by conducting pathological investigations on archived and new cases. This report describes the gross and light microscope characteristics of the hyperplastic mucous glands of affected frogs and compares them with those of normal frogs. This is the first description of adenomatous hyperplasia (AH) of the mucous glands in amphibians.

MATERIALS AND METHODS

Epidemiology

All available datasheets from the Department of Conservation and Auckland Zoo (Auckland, New Zealand) were reviewed to collate information about when and where the lesions appeared, their duration and where possible, a description of the lesions at each examination. To assess if AH affected survivability, the data was analysed using a Fishers exact test using WINPEPI statistical programme (v. 11.20) (http://www.brixtonhealth.com/pepi4windows.html).

Skin sample collection

Biopsies

Biopsies of skin sample collectoion of skin lesions were taken from four frogs from Auckland Zoo in April 2008 and one frog from the University of Otago in May 2007 (Dunedin, New Zealand). Two of the frogs (A60157 and A60151) were from the wild Whareorino forest population and had been in captivity for 18 months at Auckland Zoo with lesions of two months duration. Two other frogs (A50092 and A50108) were from the Coromandel population, had been captive for about seven years and had developed the lesions while at Canterbury University (Christchurch, New Zealand) at least three years earlier. The frog from the University of Otago (HZQ95) came from the wild Whareorino forest population and had been in captivity for four months with lesions of two weeks duration.

Frogs were anesthetized individually in a two litre glass chamber with oxygen at a flow rate of two litres/minute and isoflurane at 5% (VCA; Blacktown, Australia). Induction took 3-9 minutes. Heart rate was monitored via a Doppler stethoscope (Vasculoscope Model 820, Kamiga Tsusan Kaisha Ltd., Tokyo, Japan). Frogs were positioned on their dorsal side under a dissecting scope (Leica EZ4D; North Ryde, Australia) for the procedure. Skin biopsies were taken using sterile surgical scissors and removing a 1-2 mm x 1-2 mm full thickness skin sample of the lesion. Half the sample was placed in 10% neutral buffered formalin and the other in 2.5% buffered glutaraldehyde (2.5% glutaraldehyde in 0.15M sodium cacodylate buffer with 2 mM $CaCl_2$ buffer; pH 7.3). If a second biopsy was taken the sample was frozen in a -150°C cryofreezer (Sanyo Ultra Low MDF; Panasonic; Macquarie Park, Australia). The skin was closed with 6-0 Novafil (Covidien; Dublin, Ireland) using one or two simple interrupted sutures. The procedure and anaesthetic plane lasted seven minutes. The frogs took 7-10 minutes to recover in room air or in 21% oxygen in the anaesthetic chamber. Frogs received ketoprofen 100mg/ml (Troy; Glendenning, Australia) at 1mg/kg (percutaneously once a day for three days) for post-operative pain relief and enrofloxacin 25 mg/ml (Bayer; Auckland, New Zealand) at 10mg/kg (percutaneously once a day for 14 days) to prevent infection.

Post-mortem samples

Prior to 2008, frogs that died in capitivity were placed whole in 10% neutral buffered formaling and embedded whole in paraffin and processed for histology. From 2008-2010, skin samples were taken opportunistically at necropsy from 37 frogs (11 had lesions present) from 15 minutes to 24 hours post-mortem. Sharp sterile scissors were used to dissect a 1-2 mm x 1-2 mm piece of ventral caudal abdominal skin. All samples were placed into 10% neutral buffered formalin. In two cases (A60160 and A50056) where frogs had always been reported as having normal skin, samples were taken within a half hour post-mortem and a small sample of skin placed in 2.5% buffered glutaraldehyde for TEM.

Histology

All histological samples were prepared using routine methods and stained with haematoxylin and eosin (H&E), periodic acid schiff (PAS) and in some cases Ziehl-Neelsen (ZN), at either Massey University (Palmerston North, New Zealand) or Gribbles Veterinary Laboratories (Auckland, New Zealand). The mucous glands were examined and measured using a light microscope with a camera (Leica DME 1395XXX/Leica EC3, North Ryde, Australia). The following categories were designed to characterize the mucous glands from both apparently normal and affected frogs to enable consistent description: size of glands (width x depth); location in skin (epidermis, dermis; superficial or deep); organization (well organized (layers around a central lumen) versus disorganized (no lumen, no layers)); circumscribed; nuclear to cytoplasmic ratio; cytoplasmic appearance (vacuoles, amount of cytoplasm, colour of cytoplasm); nuclear appearance (anisokaryosis, colour); nucleoli (visible and number); mitotic figures/ hpf (400x). The descriptive data was collated but only the biopsied frogs were used to obtain measurements (Table 1).

Transmission electron microscopy (TEM)

Skin samples were placed in the primary glutaraldehyde fixative as above overnight at room temperature. The tissue was then washed in 0.15M sodium cacodylate buffer, post-fixed in 1% OsO_4, washed again in 0.15M sodium cacodylate buffer, dehydrated and infiltrated in epoxy resin (Procure

812; Thuringowa, Australia). Flat blocks were cut with an ultramicrotome (Leica Ultracut UCT ultramicrotome; North Ryde, Australia) into semi-thin (1µm) and ultrathin (70-90nm) sections. The former were stained with methylene blue for preliminary light microscope observations. Ultrathin sections were collected on 150 uncoated mesh copper grids and formvar coated slot grids and stained with uranyl acetate and lead citrate. Samples were viewed under a transmission electron microscope (FEI Tecnai G^2 Spirit Twin 120Kv; Hillsboro, USA).

RESULTS

Epidemiology

Between 2000 and 2012, AH was recorded in 35 of 94 (37%) frogs at all three institutions (Auckland Zoo, the University of Canterbury and the University of Otago), in frogs collected originally from both the Coromandel and Whareorino forest populations. Lesions appeared after between four months and nine years in captivity, and in some cases occurred in frogs that had never been held with affected frogs. At Auckland Zoo the lesions occurred in eight of the ten enclosures.

Twelve of the 35 frogs that had AH were still alive in 2012, and in nine of these survivors the lesions had resolved completely.

We compared the risk of death between those frogs where AH present and those that had normal skin. The relative risk of death if AH present was 0.9 ((C.I. 95% 0.7-1.2), Fishers exact 1 tail test P=0.9) which is biologically insignificant.

Gross pathology

In frogs with normal skin the ventral skin was pigmented with a smooth, moist surface.

In frogs with abnormal skin the lesions varied in appearance. The size of each lesion ranged from <0.5-1.5 mm. Most were papules that were circular or oval, regular in outline, and dome shaped

with no umbilication. The overlying epidermis was not fragile and there was no associated inflammation. Contents often appeared clear or semi-transparent. Others appeared as raised papules or plaques covered by normal appearing skin. Lesions were located predominantly on ventral surfaces including trunk, thighs, lower legs and forearms, but not on digits. The number of lesions ranged from a single lesion to multiple lesions covering the entire ventral surface of the frog. In some cases the lesions were difficult to see since they were not prominent and the multiple pale patches forming part of the normal colour pattern of the frog tended to obscure small pigmented lesions (Figure 1a-f).

Histology

In normal frogs, mucous glands averaged 66 µm in width x 19 µm deep (orientation superficial to deep dermis) and were located in the superficial dermis. Each gland is connected to the surface of the skin by an epidermal duct (Melzer et al., 2011). The glands were well organized with one cuboidal to attenuated epithelial cell layer surrounding a central lumen of 40 x 10 µm. The glands were well circumscribed but not encapsulated. The nuclear to cytoplasmic ratio was approximately 3:1. Cells had scant, moderately basophilic cytoplasms with rare vacuoles. Nuclei had mild anisokaryosis (round to oval; 5-9 µm diameter) and were deeply basophilic with rare nucleoli. Mitotic figures were absent or rare.

The characteristics of the glands with adenomatous hyperplasia have been summarized in Table 1. The abnormal glands from skin biopsies averaged from 491 µm in width to 370 µm in depth (orientation superficial to deep dermis). The glands were enlarged to fill the entire dermis, elevating the epidermis and compressing the deep dermis. All the biopsied glands appeared semi-organized. They varied from having a lining of attenuated epithelium and multiple cystic areas to having sheets of cells with acinar structures with central lumina 30 to 145 µm in diameter. Some lumina contained lightly basophilic material which was PAS positive - consistent with mucus secretions (Brizzi et al., 2002; Fontana et al., 2006) (Figure 2a-d). The glands were circumscribed but not encapsulated. The

nuclear to cytoplasmic ratio was 1:1 or 1:2. Glandular cells had moderate, lightly basophilic cytoplasm and some cells had cytoplasmic vacuoles. Nuclei had moderate anisokaryosis (some clefted; 5-13 µm in diameter) and were mildly basophilic. Usually one nucleoli was visible and most nuclei had clumped chromatin. Mitotic figutes were rare (0-3mitotic figures/hpf) (400x magnification).

Transmission Electron Microscopy

The epidermis and basement membrane were intact. No viral inclusions, protozoa, bacteria or fungi were seen in the epidermis or normal mucous glands. No subcellular abnormalities were observed in the affected epithelial cells (Figure 3).

DISCUSSION

The enlarged proliferating non-invasive lesions described in the dermal glands in this study have been termed adenomatous hyperplasia (AH). This is consistent with the use of the term to describe the crowded adenomatous epithelial nodules that occur in many glandular tissues throughout the body (e.g. uterus, prostate, pancreas and thyroid gland) in a variety of species and in some cases may predispose to neoplastic transformation (La Perle, 2012). The lesions are often termed multifocal nodular hyperplasia but in this case the lesions were too small to be classed as nodules. The syndrome described here was not considered to be neoplastic based on the reversible nature of the lesions and their histological characteristics.

We were unable to determine any cause of the adenomatous hyperplasia using the clinical, pathological and epidemiological information currently available. It is not consistent with any known infectious disease of amphibians. The lesions had some of the characteristics of sebaceous hyperplasia in dogs (Goldschmidt M.H. and M.J., 2008) but there was no evidence that they were age-related. Skin lesions associated with viral diseases, such as papilloma viruses, typically progress through a sequence of development that takes several days to weeks and usually have an inflammatory

component which was not observed in these frogs (Hamada et al., 1990). The blisters in bullous pemphigoid typically have the plane of separation just above the basement layer of the epidermis, lack a significant inflammatory response, and do not progress (Chaidemenos et al., 1998; Yancey, 2005). These characteristic changes were not found microscopically; thereby ruling out many of our initial differential diagnoses for blisters. However, as we were unable to obtain TEM of the abnormal mucous glands, further ultrastructural examination is occurring post-thesis.

Analysis of the relative risk of death between those captive frogs where AH was present and those that had normal skin did not show a significant difference. However, as the hyperplastic glands lost their glandular structure and did not stain positive for mucus, disruption to cutaneous functions appears likely where widespread areas of skin were affected. The evaluation of health and mortality was confounded by the presence of metabolic bone disease (MBD) and suspect fluorosis in varying degrees in all the captive populations (Shaw et al., 2012a).

The epidemiological data also demonstrates the transitory nature of the disease, with some frogs having lesions that disappeared and reappeared or changed in number and size (Figure 1c-f). Evidence suggests the disease is unlikely to be primarily genetic and may have an environmental cause due to contact - it has only been verified in captive frogs and the hyperplasia of the glands was usually ventral in location, transitory and changing in location and size over time. Nevertheless, traumatic, degenerative or metabolic causes cannot discounted as contributing factors. Frog skins are highly permeable making them prone to environmental pollutions (Odum and Zippel, 2008). In some fish, fluorosis causes an increase in the number of epidermal mucous glands in the gills (Neuhold and Sigler, 1960). However, although Auckland Zoo had a history of fluorosis in these frogs, this current syndrome of adenomatous hyperplasia started at Canterbury University which did not have any evidence of fluoride exposure to their collection (Shaw et al., 2012a). In addition, the individual mucous glands in these cases are hyperplastic, not simply more numerous. Another environmental pollutant in aquatic frogs and fish that has been reported to cause an increase in mucus production is

ammonia, but in those cases the glands were not hyperplastic and therefore different to the AH we describe in the present cases (Lang et al., 1987; Whitaker, 2001). However, since the aetiology is still unknown and there are chemicals which can affect mucous glands in fish, it is possible that an unknown toxin or husbandry imbalance was present at all institutions in which the affected frogs resided. It is not known if physiological disruption due to MBD affected the mucous glands.

Since the frogs at Auckland Zoo have been moved to an outside enclosure with new soil, an improved diet (Shaw et al., 2012b) and consistent ultraviolet-B exposure (Shaw et al., 2012a), the adenomatous hyperplasia has resolved in most animals. This response to improved management supports an environmental cause. We recommend that further analyses of environmental parameters take place with the minimum being basic monthly substrate and water analyses (Odum and Zippel, 2008; Whitaker, 2001) and that AH continues to be investigated on the epidemiological and microscopic level to determine its cause.

Acknowledgments: The authors thank the following people for their technical assistance Dr. Lee Skerratt from James Cook University; Dr. Giovanni Delfino from the University of Florence; Dr. Richard Jakob-Hoff, Melanie Farrant and Dr. John Potter of the New Zealand Centre for Conservation Medicine; Dr. Brett Gartrell, Dr. Richard Norman and Dr. Kerri Morgan from Massey University IVABS; Matthew Downing and Sabine Metzer of the University of Otago; and Hilary Holloway from the University of Auckland. We also thank the following for their support: Peter West, Andrew Nelson and Richard Gibson from the Auckland Zoo; Lisa Daglish, Peter Gasson, Amanda Haigh and Oliver Overdyk from the Department of Conservation. Finally, the authors acknowledge the following iwi for their continued support of native frog research and conservation: Hauraki Maori Trust Board, Kinohaku West H1B1 & H1B2 Trust, Marokopa Marae Committee, Mataora No 1 & 2 Block Inc, Moehau Nga Tangata Whenua, Ngati Hei Trust, Ngati Huarere, Ngati Maru ki Hauraki Inc, Ngati Paoa Trust, Ngati Pukenga ki Waiau Society, Ngati Rahiri Tumutumu, Ngati Rongo U Charitable Trust, Ngati Tai Umupuia Te Waka Totara Trust, Ngati Tawhaki, Ngati Whanaunga, Te Kupenga O Ngati Hako Inc, Te Patukirikiri Iwi Inc, Te Runanga O Ngati Pu, Te Ruunanga A Iwi o Ngati Tamatera, Te Uringahu Ngati Maru Manaia.

Funding for TEM and histology was supplied in-kind by James Cook University, the New Zealand Centre of Conservation Medicine, the New Zealand Department of Conservation, the University of Auckland and the University of Otago.

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Table 1: Histological characteristics of adenomatous hyperplasia in post-mortem and biopsy samples. All lesions were circumscribed and filled the entire dermis.

Frog ID and sample type	Gland width (µm)	Gland depth (superficial to deep) (µm)	Size of lumen (µm)	Number of lumens	Organization (well, semi or poor)	Nuclear to cytoplasmic ratio	Cytoplasmic appearance (amount and colour)	Nuclear appearance and size (µm)	Nucleoli visible?	Number of mitotic figures /hpf (400x)
A50108 biopsy	684	300	30-45	multiple	semi: mix of sheets of cells and glandular structures	1:1 - 1:2	moderate; moderate vacuolation	moderate anisokaryosis, some clefted; bi-nucleated present; lightly basophilic; 5-9	occasionally visible; 1-3	2
A60157 biopsy	740	520	35-160	multiple irregular	semi	1:1 - 1:2	moderate; moderate vacuolation; lightly basophilic	mild to moderate; some attenuated; few clefted; moderate to lightly basophilic; 8-16	rarely visible; 1	0
A60151 biopsy	230	285	23-65	multiple irregular	Semi	1:1 - 1:2	moderate; moderate vacuolation; lightly basophilic	moderate anisokaryosis; mildly basophilic; 9-13	rarely visible; 1	0
A50092 biopsy	560	n/a	30-35	multiple round to irregular	Semi	1:1	mild to moderate; lightly basophilic	moderate anisokaryosis; mildly basophilic; 9-12	rarely visible; 1	1
A50108 post-mortem	360-745	202-420	35-264	none to multiple	well to poor	2:1- 1:2	mild to moderate; moderate vacuolation; lightly basophilic	mild to moderate anisokaryosis; bi-nucleated present; moderately basophilic; 7-13	occasional; 1-2	0
A60157 post-mortem	220-700	70-670	n/a	none	poor	3:1- 1:1	mild to moderate; moderate vacuolation; lightly basophilic	moderate anisokaryosis; mildly basophilic; rare clefting; rare nuclear clearing;	occasional to moderate;1	0

								6-12		
A60151 post-mortem	500-820	375-700	40-60	none to multiple round to irregular	semi to poor	1:1	mild to moderate; lightly basophilic	moderate anisokaryosis; mildly basophilic; 6-15	rare; 1	0
A50045 post-mortem	1161	868	n/a	none	Poor	1:1	mild to moderate; lightly basophilic	moderate anisokaryosis; mildly basophilic; 6-12	rare; 1	0
A50038 post-mortem	1180	617	40-110	multiple with PAS positive substance	well	1:1	mild to moderate; lightly basophilic	moderate anisokaryosis; mildly basophilic; 7-17	occasional; 1	1
A50111post-mortem	420-860	460-650	30-430	multiple with PAS positive substance	Well	1:2-2:1	mild to moderate; mild vacuolation; lightly basophilic	moderate anisokaryosis; mildly basophilic; 9-11	none	0
A50113 post-mortem	650	280	n/a	none	Poor	1:1-2:1	scant to mild cytoplasm; lightly basophilic	moderate anisokaryosis; mildly basophilic; 10-13	moderate;1	0
A50110 post-mortem	265	90	17-20	multiple	Semi	1:4- 2:1	scant to mild; lightly basophilic and vacuolated	moderate anisokaryosis; mildly basophilic; 7-12	occasional; 1	1
A50078 post-mortem	500	170	50-200	multiple irregular	Semi	2:1- 1:1	mild to moderate; lightly basophilic	mild anisokaryosis; moderately basophilic; 6.5-12	no	0

Figure 1. Ventral skin in adults of *Leiopelma archeyi* with adenomatous hyperplasia (a) Case A60151 with severe, widespread lesions (b) Case A50246 with fewer, subtle, pigmented lesions(c) Ventral gular region in case A60151 prior to biopsy, April 2008 (d) Same region in case A60151 at post-mortem, December 2008; circles indicate where lesions have resolved (e) Ventral gular region in case HZQ 95 with multiple lesions, May 2007 (f) Same region in case HZQ 95, with less lesions but one is enlarged, May 2012.

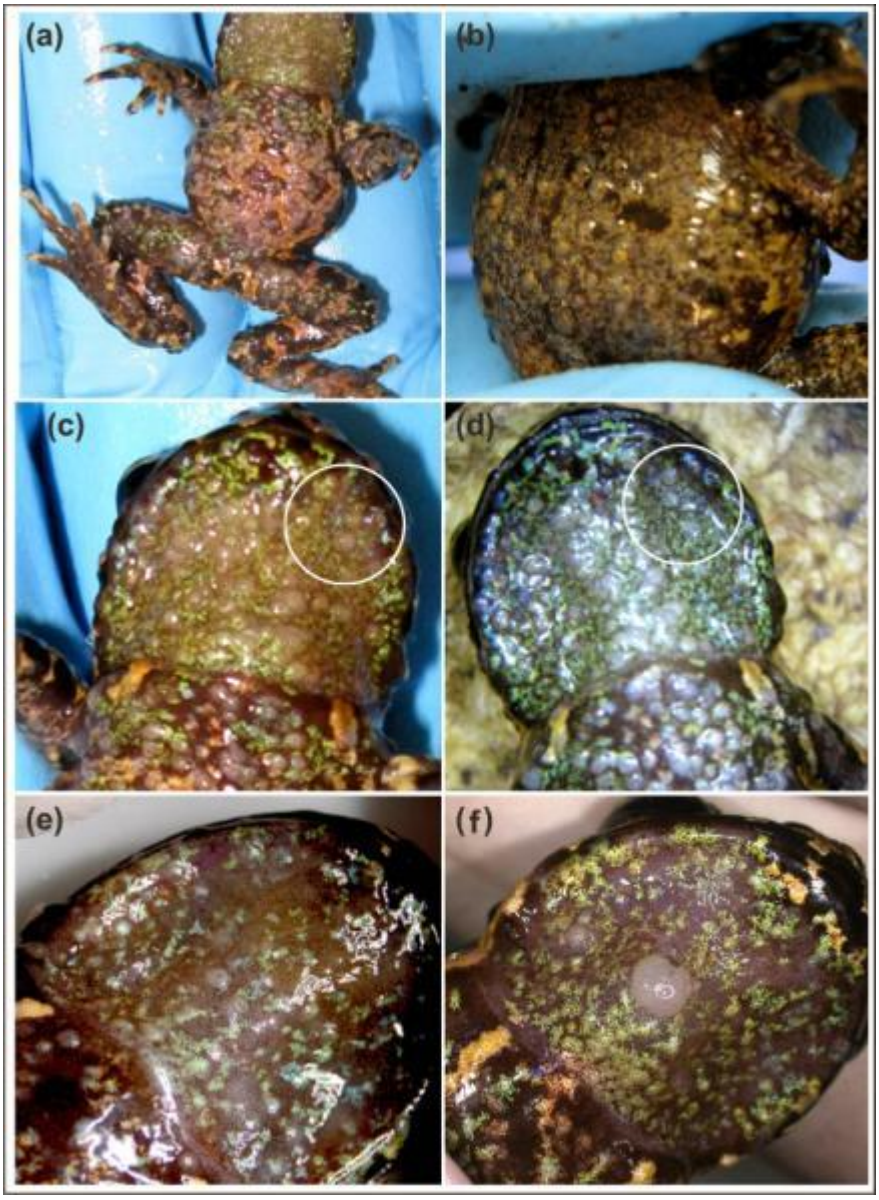


Figure 2. Histological sections of ventral skin in adults of *Leiopelma archeyi* (except as noted) with and without adenomatous hyperplasia (a) Post-mortem sample from a free-living *Leiopelma pakeka* with normal mucous and serous glands, H&E 400x (b) Post-mortem sample from a bycatch free-living *L. archeyi* with normal mucous glands- note has suboptimal preservation, H&E 400x (c) Skin biopsy of case HZQ 95 with normal (M) and hyperplastic (AH) mucous glands, H&E 100x (d) Skin biopsy of case A50108 with a well organised hyperplastic mucous gland, PAS 100x (e) Post-mortem sample of case A50111 with arrow indicating PAS positive area, 400x (f) Post-mortem sample of case A50045 with poorly organised hyperplastic mucous gland, PAS 400x

M= normal mucous gland, S= normal serous/granular gland, AH= hyperplastic mucous gland E= epidermis, D= dermis.

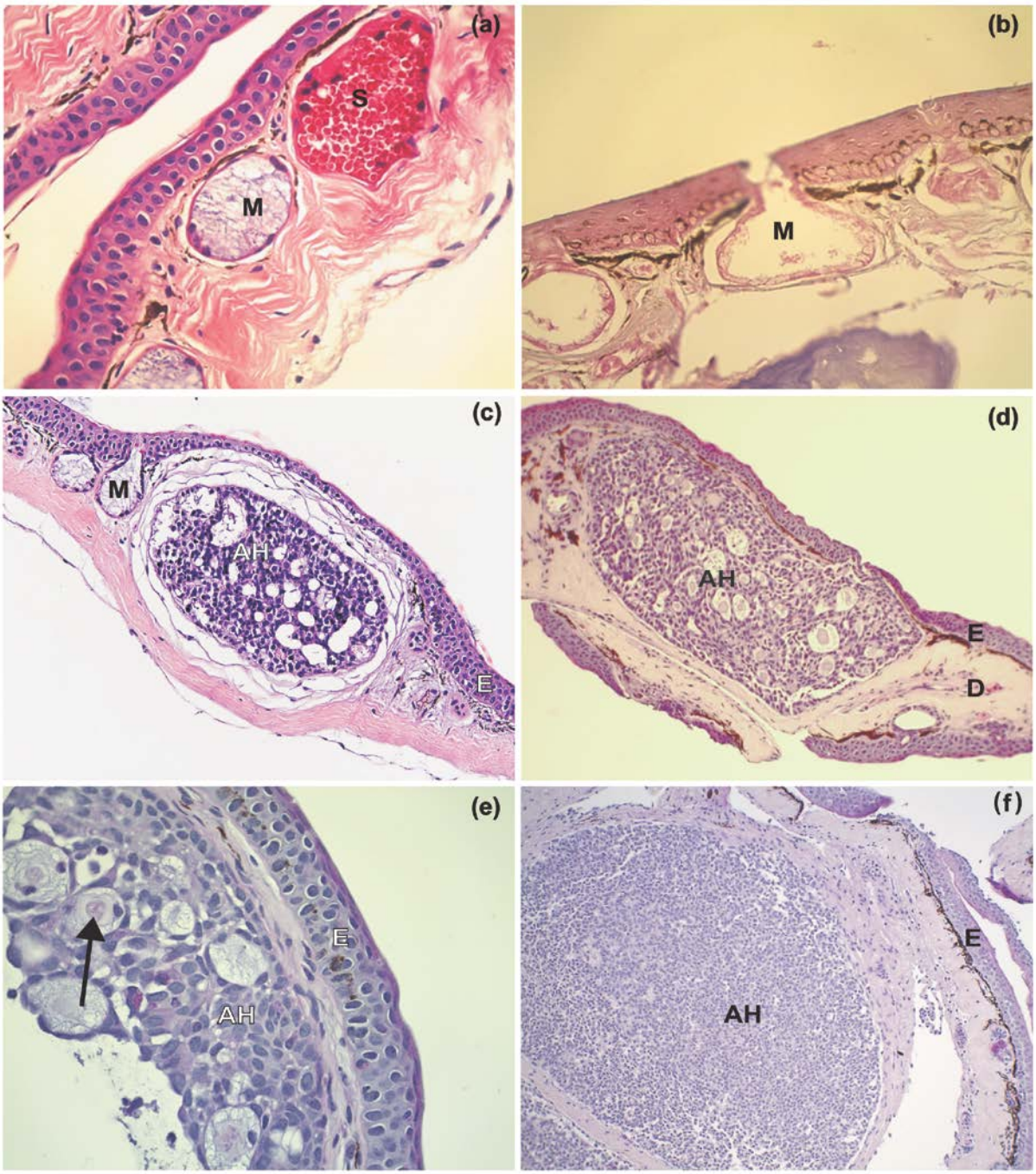
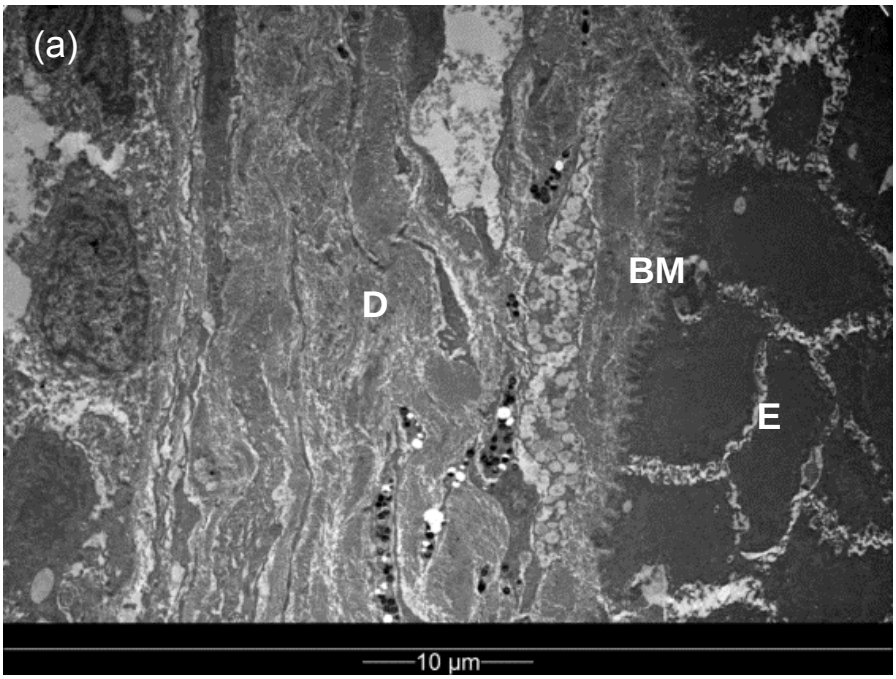
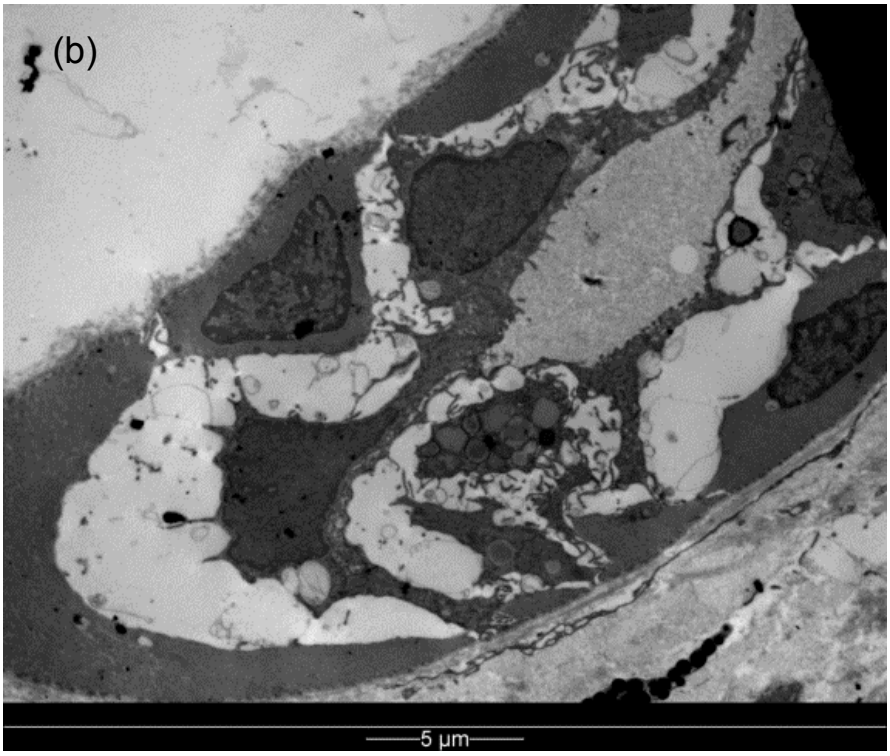


Figure 3: Transmission electron microscopy sections of ventral skin biopsies in adults of *Leiopelma archeyi* (a) Case A50108 showing normal epidermis, basement membrane and dermis. Basal epidermal cells exhibit normal features showing a comb-like profile at the dermal interface. (b) Case A60151 showing a normal mucous gland in an affected frog.

E= Basal epidermal layer D= Dermis BM= basement membrane





Chapter 7: Where have the all the frogs gone? Historical amphibian population trends based on New Zealand public observations

Preamble

This chapter starts the second half of the thesis which focuses on the wild populations of amphibians in New Zealand in order to answer the question: Is the amphibian chytrid a threat to free-ranging native frogs?

Here I describe an amphibian population survey that first arose from hearing many frog enthusiasts, laypeople and biologists alike, comment that the frogs had declined and largely disappeared in comparison to when they were growing up in New Zealand. This survey was a technique to collect long-term data from citizens about the introduced frog populations (ie, *Litoria* spp.) that were easy to see and hear, unlike the native leiopelmatid frogs. Respondents used written records they had collected over the years or used their recollections to describe the location and status of populations of *Litoria* spp.. We hoped this data could be used to either confirm or deny the assertion made by many that frogs populations in New Zealand were in decline.

This is a unique chapter in this thesis as it focuses on the three non-native frog species in New Zealand (*Litoria aurea*, *Litoria ewingii* and *Litoria raniformis*). Here I used citizen science to obtain qualitative historical data about population trends of frogs. The aims of this chapter were to:

1) test the anecdotal rumours that frogs in New Zealand are in decline and if so, to identify the location and timing of any declines and any associated factors;

2) identify growing or stable populations of *Litoria* spp. which could assist future disease surveys or population monitoring, and to identify sources of genetic material that may serve as an Ark for declining Australian populations; and

3) identify suitable regions for translocations of *Leiopelma* spp. where *Litoria* spp. populations were not reported to reduce the risk of disease transmission from non-native to native species.

This chapter was written to be submitted to the New Zealand Journal of Zoology and is ready to submit post-thesis with formatting changes.

My contribution: 80%. Lee Skerratt and I formulated the 2008 survey and did the statistics together. I reviewed and collated all the surveys including those originally from Phil Bishop. As indicated, I used 44 of Dr. Bishop's original surveys from 1998 after reviewing hundreds which were not useable. Joel Myhre created all the GIS maps following my directives. Rick Speare and Lee Skerratt both contributed to the editing of the manuscript.

Where have the all the frogs gone? Historical amphibian population trends based on New Zealand public observations

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ABSTRACT: Surveys were distributed to New Zealand land users in 1998 and 2008 to acquire information about the distribution and population levels of both native (*Leiopelma* spp.) and non-native *(Litoria* spp.) frogs. Overall frog populations in New Zealand were reported as declining, but were actually stable or increasing in a few regions. Possible causes given for declines were an increase in agriculture, increase in the distribution of predatory fish and disease. The distribution of *Litoria* spp. was from Kaikohe in the Northland region to Invercargill in the Southland region. The distribution maps could be used for three main purposes: 1) to identify suitable regions for translocations of *Leiopelma* spp. where *Litoria* spp. populations were not reported to reduce the risk of disease transmission from non-native frogs to native frogs; 2) to identify growing or stable populations of *Litoria* spp. which may assist future disease surveys, population monitoring and to identify sources of genetic material that may serve as an Ark for declining Australian populations; and 3) to confirm the anecdotal rumours that frogs in New Zealand are in decline and if so, to identify the location and timing of any declines and any associated factors. This will highlight hotspots for detailed disease studies.

KEYWORDS: amphibian · chytridiomycosis · *Leiopelma* · *Litoria* · New Zealand · survey

INTRODUCTION

In New Zealand if you work with frogs, chances are the baby boomer generation and older will tell you "the frogs used to be around when I was a kid, but now they have all disappeared". Using questionnaires to survey knowledgeable people about animal disease and movements to acquire quantifiable evidence can provide valuable data that is otherwise difficult to obtain. For example, the distribution of sarcoptic mange in wombats was mapped by surveying animal caretakers and biologists in Australia as to where they had seen the disease (Martin et al., 1998). In New Zealand, forest plantation workers were surveyed to help map the locations and the use of forests by New Zealand long-tailed bats (Borkin and Parsons, 2010). This type of data collection is called "citizen science" and is a well-established method to enable researchers to collect large amounts of data over a geographic area where it may otherwise be prohibitive in terms of cost or manpower (Ashcroft et al., 2012; Kadoya et al., 2009; McCaffrey, 2005; Swengel, 1990). Surveying land users in New Zealand then may be a useful tool to obtain information about the health and locations of our frog populations.

There are four species of native leiopelmatid frogs and three species of introduced hylid frogs in New Zealand. Their conservation status and population levels have been in the spotlight for the past decade since the discovery of chytridiomycosis as a cause for both local and worldwide amphibian declines (Berger et al., 1998; Bell et al., 2004; Lips et al., 2003; Skerratt et al., 2007). According to the International Union for Conservation of Nature and Natural Resources (I.U.C.N.) Red List of Threatened Species (2011) (Accessed 20 March 2012; www.iucnredlist.org) , the population levels and stability of New Zealand's native amphibians are rated as follows: *Leiopelma archeyi* critically endangered/decreasing; *Leiopelma hamiltoni* endangered/stable; *Leiopelma hochstetteri* vulnerable/unknown; *Leiopelma pakeka* vulnerable/stable. The three introduced *Litoria* spp. living in New Zealand, but rated according to their endemic Australian populations, are as follows: *Litoria aurea* vulnerable/decreasing; *Litoria ewingii* least concern/stable; *Litoria raniformis* endangered/ decreasing. *Litoria* spp. were introduced into New Zealand from Australia in the 1860's (Pyke and White, 2001; Voros et al., 2008) and as such are not offered any legislative protection in New Zealand. However, members of the public often see them as "New Zealand" frogs and go to

great lengths to monitor and improve their survival. For example, many create protected ponds in their gardens to increase frog habitat and some even create new populations for their enjoyment. The people who monitor these frogs on a year to year basis may have historical information that is irreplaceable. Anecdotally these land users have reported mass population declines in *Litoria* spp. in New Zealand, but field studies have not been done to document the supposed declines or any associated causes.

One known cause of worldwide amphibian declines is chytridiomycosis (Skerratt et al., 2007). Chytridiomycosis is a disease caused by the amphibian chytrid fungus, *Batrachochytrium dendrobatidis* (Bd) (Berger et al., 2005; Longcore et al., 1999). The three *Litoria* spp. present in New Zealand are moderately susceptible to chytridiomycosis (Berger et al., 2004a; Obendorf and Dalton, 2006; Stockwell et al., 2010; White, 2006) and the disease has been documented in all three species on both the North and South Islands (Shaw et al., 2009; Waldman et al., 2001). Although local die-offs in New Zealand caused by chytridiomycosis have been documented in *L. aurea* and *L. raniformis* (S.Shaw, unpubl. data; Waldman et al., 2001), at present *Litoria* spp. are not monitored in New Zealand; so their current numbers and the effect of chytridiomycosis on population levels are unknown. In the leiopelmatids it has been shown that captive Archey's frogs infected with the amphibian chytrid can self-cure (Bishop et al., 2009; Shaw et al., 2010) and that *L. pakeka* may also be able to self-cure (Ohmer, 2011). However, as previous exposure to Bd can't be ascertained, the laboratory studies could not prove that any naïve *Leiopelma* spp. populations, if they exist, are not still at risk to population crashes from chytridiomycosis as is thought to have occurred to the Coromandel population of *L. archeyi* in 1996 (Bell et al., 2004). Other threats to both *Leiopelma* and *Litoria* spp. could be predation, habitat depletion or degradation (e.g. mining), exotic disease (e.g. Ranaviral disease) and chemical exposure (Bell et al., 2004; Daszak et al., 1999; Pyke and White, 2001).

Therefore, in 1998 a frog report was designed to obtain an accurate distribution record of *Litoria* spp. around New Zealand by collecting sighting data from both scientists and the general public (Bishop, 1999). This data was added to the Department of Conservation Herpetofauna Database and the results mapped to give an updated distribution map (Bishop, 2008). In 2008, we

modified and expanded the survey to inquire specifically about long term population data, rather than one-off sightings. The goal of this study was to collate the answers from both surveys to assess if we could accurately compile and map the distribution and population trends of amphibian populations without costly and time-consuming field surveys. The maps produced would give different but complementary information on frog populations in comparison to the simple distribution of single frog sightings that the Herpetofauna Database produced (Bishop, 2008). The information from these surveys could be used for three key objectives: 1) to identify suitable regions for translocation of *Leiopelma* spp. where *Litoria* spp. populations were not reported. This will reduce the risk of disease transmission from non-native to native species (Bishop and Germano, 2006; Germano and Bishop, 2009); 2) to identify growing or stable populations of *Litoria* spp. which may assist future disease surveys, population monitoring and to identify sources of genetic material that may serve as an Ark for declining Australian populations; and 3) to confirm the anecdotal rumours that frogs in New Zealand are in decline and if so, to identify the location and timing of any declines and any associated factors. This will highlight hotspots for detailed disease studies. In addition, although intense field surveys are already in place for New Zealand native frog species, the identification of declining non-native frog populations may identify unknown threats to the native frog populations.

MATERIALS AND METHODS

In 1998, a "Frog Observation Form" was formulated as part of the New Zealand Frog Survey (Appendix 1). It was distributed to schools, the Department of Conservation, and herpetology clubs. The survey had six pages of background information and one form to be filled in with 17 specific questions. Fifteen of the questions were open questions asking contact details, map grid location and locality where the frog was sighted, the species of frog, weather data (air temperature, cloud, wind and rain), habitat type, microhabitat description and any land changes noticed. Two questions were tick boxes about frog behaviour and life stage. Surveys were collected from 1998 until 2006. When analysing those forms for this study only reports that had all data fields completed were used. In addition single sightings of a single frog were excluded.

In 2008, a new survey called the "New Zealand Frog Distribution Survey" was created to add to the data collected by the earlier survey. In order to collect new data it was designed to get data from different sources (more of an emphasis on amateur sources whereas the earlier survey had focussed on professionals) and therefore it was thought it would be likely to obtain data on different frog populations (Appendix 2). This particular title was chosen so as not to lead the respondent to thinking about population decreases only. The new survey was shorter, had mainly closed questions (tick boxes) and the questions had been modified for improved quality of responses and to be more user-friendly. A small paragraph asking people if they were interested in filling out a survey regarding frog populations in New Zealand, was published in a newspaper, the Waikato Times, and five magazines (Pet, Vetscript, Forest and Bird, Hunting and Fishing New Zealand and New Zealand Rod and Rifle) over a period of six months in early 2008. These publications were chosen to target readers using the outdoors for recreation, those working with animals and those who lived in regions with frogs to increase the number and quality of the responses. The survey was also distributed to Department of Conservation personnel known to be working with amphibians. Respondents emailed or called to ask for a survey to complete which was then emailed or posted out to them with a postage paid return envelope. Surveys were collected until the end of 2009. The 2008 survey had eight specific questions; three questions collected personal details and the rest used tick boxes to gather information about frog species, population trends, the observational time frame, climate and habitat. The location was determined by asking for a specific location name and the corresponding NZ Topographic 260 Map series 1:50,000 scale. In addition each location was assigned to the one of the sixteen New Zealand legislative regions (as defined by the Local Government Act 2002) it belonged to in for analytical purposes. They were also asked to report on any other personal observations that they believed altered frog populations and to give permission to allow them to be contacted for more information. If blanks were left or boxes not ticked, the person was contacted by telephone or email to clarify the answer. If any blanks were remaining on species, time frame, or population trend the survey was excluded from analysis. Useable population trend data in this project was defined as any time frame greater than one week with repeated sightings in the same location with more than one individual frog. Single sightings of a single frog were excluded.

Both sets of data were collated. The proportion of reports from a particular region with their population trend (increasing, decreasing or stable) was collated. The median trend was calculated and presented.

A Kappa test was performed to compare agreement of the two surveys using results from survey time frames 1970-1995 and again 1999-2006 (i.e., population trends during these time frames as these were periods of likely population change) using WINPEPI statistical programme (http://www.brixtonhealth.com/pepi4windows.html). This was done to ensure that the surveys were collecting data from different frog populations.

The types of habitat that were reported with the frog sightings was assigned to a man-made (defined as any habitat that was created by humans such as a pond, swimming pool, or water trough) or natural habitat category and collated by frog species.

All useable surveys first had the decimal latitude and longitude constructed from the reported locality names and NZ topographic map locations using the website http://itouchmap.com/latlong.html. These locations were then mapped using ArcGis (v.10). Three maps were created. The first was a simple distribution map of observed populations of all frog species reported. The second was a map showing each reported population trend result by location. The third map was created to show the population trend reported and in what year the observation started. Only *Litoria* spp. were shown in this map to reduce the number of variables and the species were not differentiated since it assumed that the three *Litoria* spp. have similar susceptibility to disease and other disturbances.

RESULTS

Forty-four questionnaires were usable from the 1998 survey for this particular study, although hundreds were received. The large majority were one-off observations which were excluded. The earliest observation from this survey was 1929 in Whitianga. Eighty-six questionnaires were returned from the 2008 land user survey. Sixteen of these did not contain a timeframe or population trends so

were excluded, leaving 70 for analysis. The earliest observation from the second survey was 1940 from Winton.

The largest percentages of the 2008 surveys were returned from the Waikato and Auckland regions at 21% and 17.2% respectively. Both the Hawke's Bay and Marlborough regions had no useable surveys returned. Six of the 14 population trend medians by region were reported as decreasing while five were stable. Two medians were midline between decreasing and stable. However the overall median was decreasing giving the overall population trend reported for amphibian populations as decreasing (Table 1).

The Kappa test between the two surveys was less than zero which is non-agreement. This result is interpreted to mean that the surveys were not about the same frog populations and could be combined to yield more results. This result of non-agreement is not surprising since most observations that people made were about one particular frog population, often on private land, and should have been different populations.

Frogs were found equally in both man-made and natural habitat (Table 2).

The distribution map (Figure 1) contains the reported locations for *Litoria aurea*, *Litoria ewingii*, *Litoria raniformis* and *Leiopelma. hochstetteri* populations. No useable surveys were returned for *Leiopelma archeyi*, *Leiopelma hamiltoni* or *Leiopelma pakeka*.

The second map (Figure 2) shows the relative change of the reported frog populations. In general, most declines were reported on the South Island on the Northwest coast from Fox Glacier to Nelson and the Invercargill region. On the North Island most declines were reported in the Auckland and Waikato regions. Most increases and stable populations were noted on the central Eastern coast of the South Island and the Waikato region and southeast coast of the North Island. There were gaps in reporting in the Marlborough region of the South Island and Hawke's Bay in the North Island.

The third map (Figure 3) shows the relative change of the frog population with the first year that trend is reported. Declines were reported in the late 1980s, 1992, 1994, 1995, 1996, 1997, 1998

and 2006 in locations on both North and South Island. Some surveys did report a decrease and then an increase which could not be depicted on the map: Kaikoura 1982-2002; West Auckland in 1985-2008; Wellington two locations 1987-1999; Port Jackson, Coromandel 1997- 2008; Tapu, Coromandel 1997-2000; Palmerston 1998-2008. The first reported population increase was *L. ewingii* in 1976. Most increases on the North Island started in 2003 although a few in the Wellington region reported increases in the late 1990s.

DISCUSSION

Both the 1997 and 2008 frog surveys indicated that frog populations in New Zealand are in overall decline. The goal of the study was to correlate the answers from both surveys to assess if we could accurately compile and map the distribution and population trends without costly and time-consuming amphibian field surveys was accomplished.

The surveys were successful in creating a database of known frog locations that were easily visualized on the maps thus addressing the first and second objectives: to show locations where frog populations have and have not been reported. As both surveys ask for frog sightings, the responses are biased towards non-native frogs which are easily seen and heard, as opposed to native frogs which are silent, nocturnal and whose habitat requirements tend to be in protected areas. Another bias could be that frogs located near where people live and visit are more likely to been seen, heard and found alive/dead. There is also the issue of data quality derived by using citizen science. In this case, we mainly published our survey participation requests in magazines whose readers were most likely to have a particular interest or skill in animal observation, thereby potentially increasing the level of quality of long-term observations. We did not question the accuracy of the responses in terms of frog identification, nor offer any specific training to those who responded to the survey. The difference between the very small, brown *L. ewingii* and the larger, green *L. raniformis* and *L. aurea* is obvious on colour and sometimes size depending on the life stage observed. Hence, for *L. ewingii* misidentification is unlikely. It is possible for people to mistake *L. ewingii* with the native *L. archeyi*, but this did not happen in our survey as the responses were carefully screened for this potential mistake. In cases where the species of *Litoria* was not clear, the term *Litoria* spp. was used. It is possible that in the areas of the North Island where *L.*

raniformis and *L. aurea* co-exist that their identities could have been mistaken, especially as they may hybridize (P.Bishop, pers. comm.). However, for the purposes of this study, it was assumed that the three *Litoria* spp. have similar susceptibility to disease and other disturbances so their exact identity was not important enough to warrant identification training prior to filling out the survey. The 1998 survey did include two pages of frog identification assistance, but in the 2008 survey the participant was referred to the website www.nzfrogs.org.nz which had all the necessary information to help identification issues. The second survey was also asking for recalled data, so training would likely to have little effect. However, specific training may be necessary for any future studies if species identification is important (Ashcroft et al., 2012).

Overall the new distribution maps appear to have less data points than the 2008 Department of Conservation Herpetofauna map (Bishop, 2008). However, as the DOC map contains one-off sightings our map is more useful as it only reports repeated observations and reduces the potential areas in which to look for established frog populations. The population trend map shows that the Auckland, Waikato and Tasman regions reported the highest number of increasing populations so those regions may also be favourable locations for field surveys to start, saving both valuable time and money. Most surveys also reported the habitat where the frogs were found and that in some cases, increases were associated with swimming pools and ponds that the public created. This sort of preferred habitat confirmation can also assist field surveys in reducing the locations to search.

These maps also show regions where frogs have not been reported. Using this information in combination with current Department of Conservation frog distribution reports of *Litoria* spp. sightings could help to narrow down sites for future *Leiopelma* spp. translocations by eliminating any site with *Litoria* spp. present. Eliminating these sites could reduce the risk of disease transmission between non-native and native frog species and the possibility of predation of *Leiopelma* spp. by *Litoria* spp. which could occur due to their size difference (Thurley and Bell, 1994).

The third objective was to verify the long-standing hearsay from the New Zealand public that the frogs in New Zealand are in decline. Our data does verify that the public have reported that most

frog populations have declined. However, if the public observation was of a long time frame (i.e. 80 years), then the actual moment of decline is not obtained with this type of reporting. The survey should have asked one more question asking for a specific year of population increase or decrease. Instead there was a space for general observations relating to the population trend that could be answered. Some responses that reported an overall decrease, actually did give a year or several years that the frogs sharply decreased or disappeared. We expected that most declines would be after 1999 when the amphibian chytrid was first reported in New Zealand (Waldman et al., 2001). However, there were some reports of decline before 1999. This leads to the second part of the last objective: "what was going on in that time frame that may have caused a decline?" Factors such as pesticide use and changing farming practices could have caused the earlier declines. Three responses reporting a decline in the 1970s remarked that increased agriculture, pesticide spraying and land clearing did cause an obvious decrease. One biologist reported that from 1990 the numbers of pest fish increased and, although the number of ponds also increased, the frogs did not. Again, as little scientific data is available documenting declines and associated causes, this information from land users is irreplaceable in looking at agents of decline. One hypothesis to explain declines prior to 1999 was that chytridiomycosis was introduced prior to that time. In the South Island, some reports of stability and increases were noted from 1971 – 2004 mainly in the Canterbury region. This is surprising as Christchurch is the first known confirmed location of chytridiomycosis, which is in the Canterbury region (Waldman et al., 2001). If Bd was introduced into New Zealand at Christchurch, it would be expected that a wave of declines in *Litoria* spp. populations would have been reported in the surveys emanating from Christchurch. As this was not the case, it could be that infected *L. raniformis* were actually introduced into that Christchurch pond from a different region from the pet trade, or that the survey data is deficient in reports from that area and the declines were just not reported. As the data from Christchurch only shows stability in 1980 and an increase in 2004, both in *L. ewingii*, the survey cannot distinguish these options. Further targeted questionnaires in the region could clarify this point and would be an important finding. Consider however the hypothesis that Bd was introduced in another port region such as Auckland in the late 1980s and released locally and spread around the country both naturally and via the pet trade. This scenario would agree with the survey data seen with

declines starting in the late 1980s and early 1990s, with Bd arriving in the Coromandel population in 1994 and spreading. This information agrees with the timeframe situation reported in *L. archeyi* but the direction of the spread in the Coromandel according to the survey data is North to South, whereas in reality it spread from South to North (Bell et al., 2004). This highlights that these maps provide a starting point for hypothesis testing. It is known that Bd was not discovered in the Dunedin region until 2008 (S.Shaw, unpubl. data) and the reported surveys in the Dunedin area suggest this was around the time of its introduction.

Conversely there were population increases and stability reported. Population increases have been previously reported in wild *Litoria* spp. as chytridiomycosis becomes endemic. Populations that have survived may stabilize and some start to recover, with periodic seasonal episodes of deaths, but no overall decline (Berger et al., 2004a; McDonald et al., 2005). This situation may have occurred in New Zealand as some surveys in Nelson, Hamilton and the Coromandel had reported a major increase in their frogs from 2005-2008. However, following the survey's completion, three of the reported increasing populations of *L. aurea* and *L. raniformis* had confirmed epidemics of chytridiomycosis (S.Shaw, unpubl. data).

Surveys of the public cannot take the place of actual fieldwork to verify locations of frogs and their population numbers, nor can tell it tell us why the frogs in New Zealand have declined. What it can and has done is to provide a low-cost frog distribution map for field researchers who are looking for these small creatures in vast and sometimes rugged terrain. These surveys can also provide indications of gross amphibian population trends and suggest factors that may be causing these trends. Further analyses to increase the robustness of this data could include combining one-off sightings with this data and using modelling techniques to predict the potential distribution of the invasive non-native *Litoria* spp. (Kadoya et al., 2009; Schmidt et al., 2010).

Ethics: This survey was approved by the James Cook University Human Research Ethics Committee permit number H2988.

Acknowledgments: Thanks to Bruce Waldman and Richard Norman for their early involvement with the initial survey. Thanks to K. Derry of the Auckland Zoo New Zealand Centre for Conservation Medicine for in-kind office support. Thanks to all the participants who took the time to fill out the

survey and share information. Thanks to all the editors of the following New Zealand publications who assisted with the survey: Hunting and Fishing New Zealand, New Zealand Rod and Rifle, Pet magazine, Royal Forest and Bird Society, Vetscript and the Waikato Times.

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Table 1: Number of surveys returned by governmental region where frogs were reported and the status of population reported.

Region	Number of responses by region	Number of populations that reported an decrease/stability/increase in frog numbers	Median
Auckland	23	13; 6; 4	decrease
Bay of Plenty	7	3; 3; 1	stable
Canterbury	9	2; 4; 3	stable
Gisborne	3	1; 2; 0	stable
Hawkes' Bay	0		n/a
Manawatu-Wanganui	7	4; 2; 1	decrease
Marlborough	0		n/a
Nelson	2	1; 1; 0	decrease/stable
Northland	5	4; 1; 0	decrease
Otago	8	2; 4; 2	stable
Southland	10	7; 3; 0	decrease
Taranaki	4	2; 2; 0	decrease/stable
Tasman	8	3; 0; 5	increase
Waikato	28	11; 6; 11	stable
Wellington	10	4; 3; 3	decrease
West Coast	10	8; 2; 0	decrease
Total	**134 ***	**65; 39; 30**	**decrease**

*The total number of answers is greater than the total number of returns as some had populations with two trends over time and both were reported here.

Table 2: Habitat types where introduced frogs were reported to be found.

Species	**Man-made habitat: swimming pools, fishponds; damp garden; farm water tanks and catchments**	**Natural habitat**
Litoria aurea	16	17
Litoria raniformis	11	16
Litoria ewingii	12	9
Total	**39**	**42**

Figure 1: The reported presence and distribution of all reported frog populations from 1929 through 2008 by location and species with genus (where known).

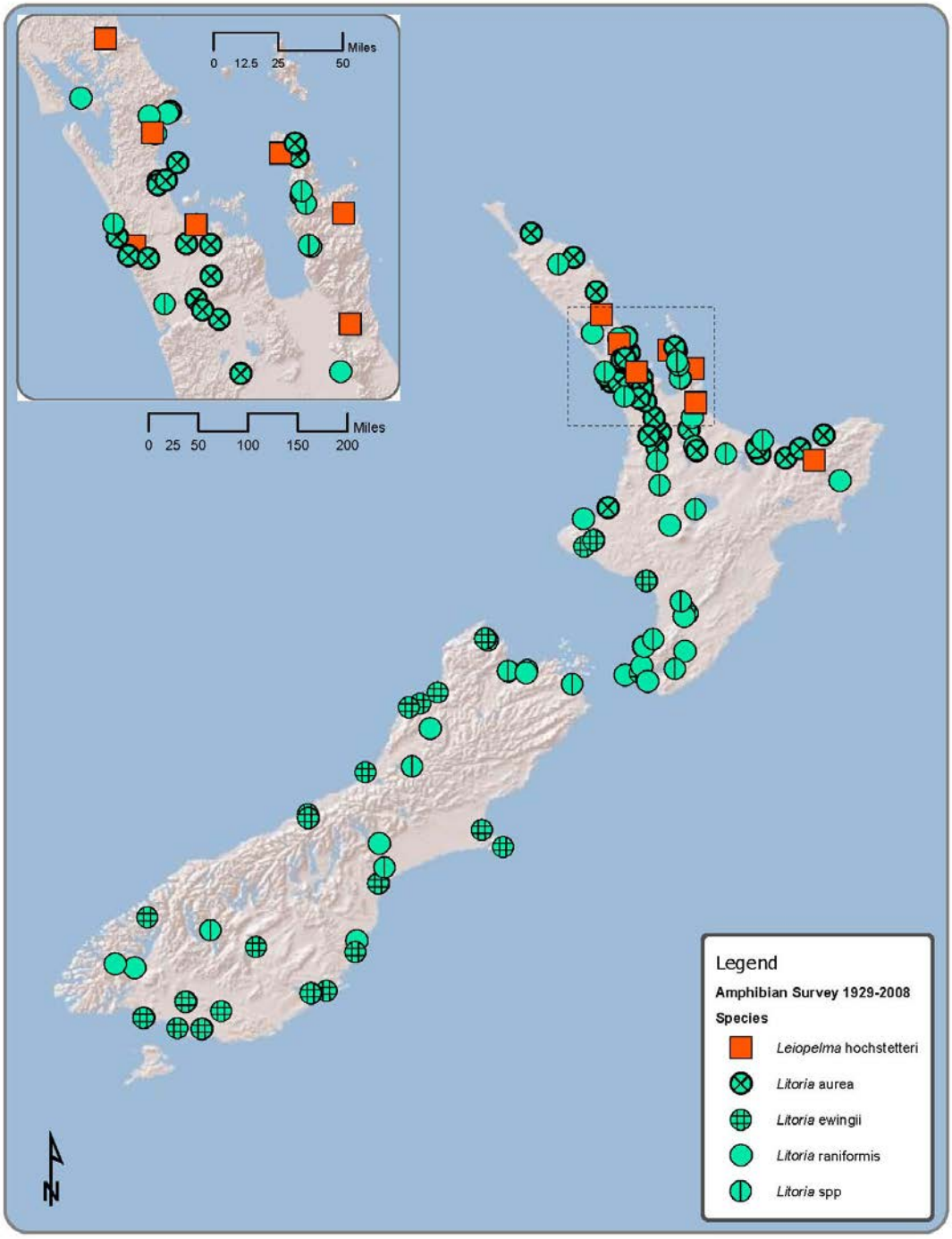


Figure 2: The reported population trend of both *Leiopelma* and *Litoria* spp. from 1929-2008 is presented by species and genus (where known), location and whether that population of frogs had been reported as increasing, decreasing or no change.

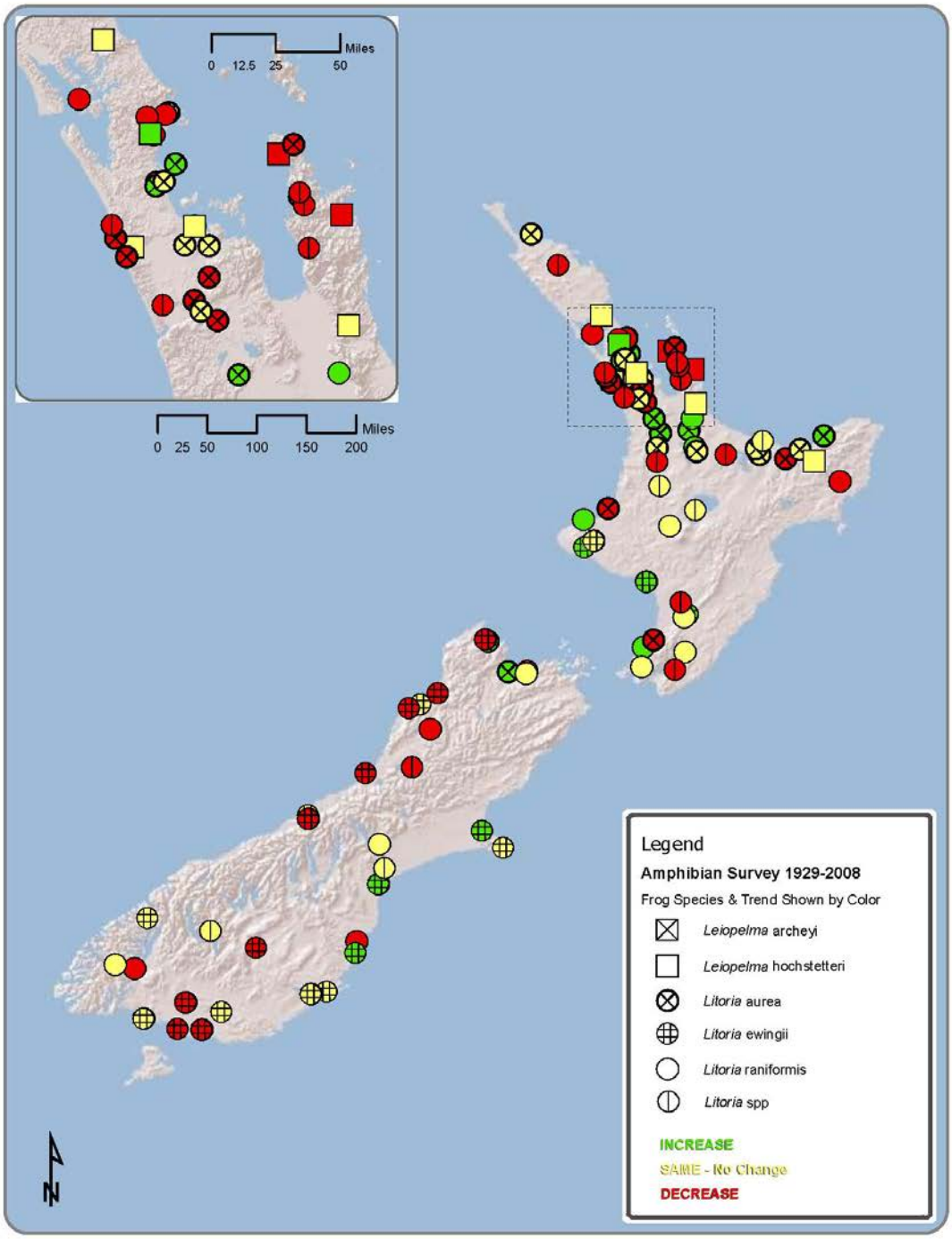
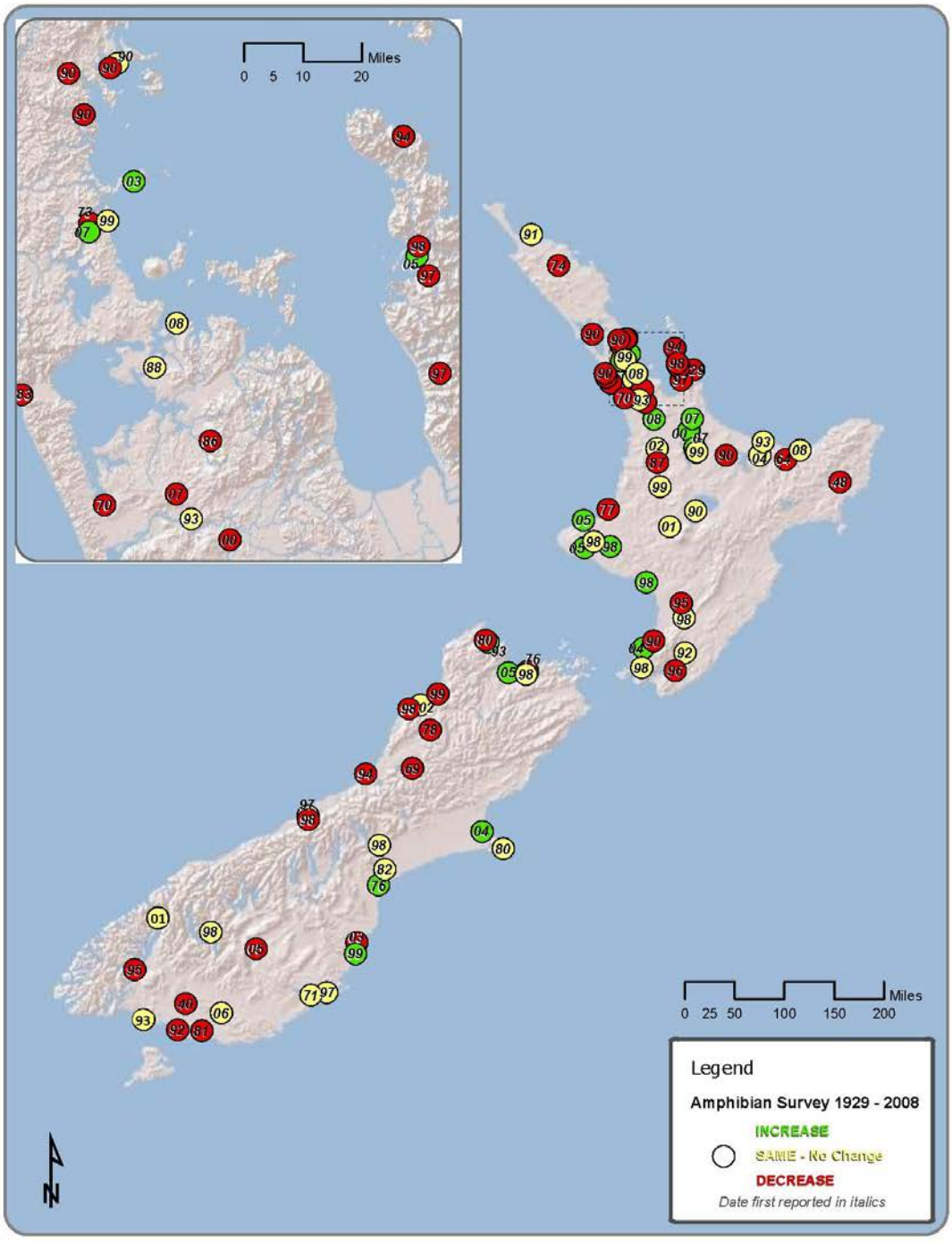


Figure 3: The reported population trend of *Litoria* spp. over time is presented by giving the last two digits of the first year the trend was noticed.



Appendices

Appendix 1. The New Zealand Frog Survey in its 1998 original format.

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New Zealand Frog Survey

January 1998

Welcome to the New Zealand Frog Survey (NZFS)!

The survey's aim is to produce an accurate and up-to-date distribution record of the frogs of New Zealand. Most frogs with which you are familiar were originally introduced from Australia over the past 100 years. Yet our knowledge of how well they are doing can tell us a lot about the health of our environment as well as potential threats to our native fauna. In this letter of introduction, we highlight the importance of Bell frogs and Whistling frogs to New Zealand, and give you information that we hope will encourage you to participate in this important area of conservation research.

Declining Amphibian Populations

Throughout the world an average of 35 species of amphibians become extinct annually. Nearby, in eastern Australia, 15 species of frogs have not been seen since the early 1980s. Some have disappeared over just the past two or three years.

Although the main cause of amphibian deaths worldwide probably is habitat destruction and fragmentation, many other causes have been implicated to varied degrees.

Forestry and agriculture take a major toll on frog populations and it has been demonstrated that roads and agricultural fields are significant barriers to amphibians.

Herbicides and pesticides often cause developmental abnormalities or fatalities. An Australian report in 1995 showed that the widely used and apparently safe herbicide "Roundup" was extremely toxic to tadpoles and adult frogs. This herbicide is widely used by farmers, foresters and gardeners in New Zealand.

Pinpointing the causes of the decline is difficult, because many causative factors interact and exacerbate the problems facing the frogs. Unfortunately, when mankind causes changes to the environment, the total effect may end up being more than the sum of the parts. For example, acidity has been shown to seriously affect the toxicity of different metals to different species of frogs. Thus acidity and pollution together may cause deaths even though neither factor alone does. Most frogs have a biphasic life cycle, where eggs, laid in water, develop into tadpoles that metamorphose into tiny replicas of the adults. This fact, coupled with their being covered by a semi-permeable skin, makes frogs particularly vulnerable to pollutants and other environmental stresses. Consequently frogs can be used as environmental biomonitors.

Indeed, they may act as an early warning system for the quality of the environment and potential threats to other animals including ourselves. Studies of the effects of pollutants and environmental change on amphibians will create a better understanding of what we need to do to protect them, and may also facilitate their use as biological indicators of ecosystem health.

One of the keys to balanced land management is an understanding of the ecological processes responsible for the support of diverse forms of life **irrespective** of their commercial value.

New Zealand and Australian Species

At present there are four species of native frogs and three species of introduced frogs in New Zealand. Two of the introduced species, the Bell frogs *Litoria aurea* and *L. raniformis*, as well as all New Zealand's unique native species (in the genus *Leiopelma),* are listed as threatened or endangered in

the 1994 IUCN Red Data Book. Hamilton's frog (*Leiopelma hamiltoni*) on Stephen's Island is one of the rarest frogs in the world with a population estimated at less than 250 individuals.

Although at least 8 species of frogs were imported into New Zealand in the late 1800s only three of these established breeding populations that still exist today. These species all belong to the family Hylidae, which includes most tree-frogs throughout the world. They are fairly easy to identify as they all produce characteristic vocalisations and appear quite distinct from the native species (see key below). The Bell frogs have suffered dramatic range contractions in Australia to such an extent that they may well be on the brink of extinction. The Green and Golden Bell frog (*Litoria aurea*) has assumed national importance in Australia, as one of its few breeding sites in Sydney is the disused brick works earmarked for the site of the new Olympic village - but only if they can create suitable accommodation for the Bell frogs elsewhere!

At present it is unknown how well the *Litoria* spp. are doing in New Zealand. A knowledge of their distribution and habits is essential if we are to attempt to unravel the mystery of declining frog populations. After a preliminary survey that we conducted during 1996, we decided that a national survey should be conducted, with the help of the public sector, to determine the presence and the status of *Litoria* spp. populations in New Zealand.

As Bell frogs are mainly diurnal feeders their main food items are grasshoppers, crickets, cockroaches, flies and even smaller *Litoria ewingii* frogs. It has been suggested that these voracious introduced frogs compete with, or prey upon, the smaller native frogs. However, the habitats utilised by the Bell frogs are not suitable for indigenous frogs and this is unlikely to be a common occurrence.

Consequently the introduced frogs are unlikely to be considered an environmental pest and may actually be helping the native species, by reducing predation pressure, as they represent an alternative, and more abundant, food supply for amphibian predators.

Litoria species in New Zealand may constitute an early warning system of the condition of the environment and hence, the impending threat to the indigenous frogs (*Leiopelma* spp.) and other native animals.

The NZFS solicits the cooperation of volunteers from schools and the public sector. We request that you submit tape recordings of frog calls, specimens, or visual identifications and photographs, together with anecdotal accounts of frog populations to us for analysis.

Introduced frogs are easy to find and identify as they produce species-specific calls enabling exact identification, without actually seeing the frog! NZFS will greatly improve our understanding of frog distributions in New Zealand and will focus attention on declining populations. The data will also be valuable for the rational planning of land-use and will support efforts to preserve our wetlands and natural biodiversity. The outcome of this project will be that frog populations can be used in a prognostic manner with respect to the condition of the environment and hence, any impending threat to other indigenous animals.

If you wish to participate in this project please complete the report form and send it together with photos or tape recordings to Phil Bishop (University of Otago, full address above) or Bruce Waldman (University of Canterbury, full address below). For colour photographs or an electronic version of the report form please visit our WEB site at **http://www.otago.ac.nz/Zoology/frogs/index.html**

Thanks for your assistance.

NZFS Coordinators: Dr Phil Bishop (address as above)

Dr Bruce Waldman

Department of Zoology, University of Canterbury,

Christchurch, Fax +64 3 3642024, Tel +64 3 3642066,

email bw@zool.canterbury.ac.nz

Frog Identification

There are three species of frogs in New Zealand which produce loud calls at and around ponds to attract females and protect individual male territories. These species belong to the genus *Litoria* and can be easily differentiated from our native **protected** species (*Leiopelma*), which are rare, essentially silent and confined to undisturbed native bush.

The key provided below is simple to use. Each question has two options and you must decide which option to follow. The number at the end of each option tells you which question to go to next. Continue to follow the correct option for your frog and you will eventually arrive at the correct identification.

Frog identification key:-

1. Frog produces a loud mating call - go to question 5.

   Frog does not produce a loud mating call - go to question 2.

2. Frog has an obvious external eardrum - go to question 7.

   Frog has no external eardrum - go to question 3.

3. Frog from nose to rear is larger than 60 mm - go to question 9.

   Frog is less than 60 mm - go to question 4.

4. Frog has the ends of its toes or fingers expanded into distal pads or suckers - go to question 7.

   Frog does not have suckers on its fingers or toes - ***Leiopelma*** species.

5. The call is a set of harsh grunts or groans - go to question 6.

N.B. - *Leiopelma* are protected by law, please do not capture or disturb them. Note on your report form their exact position and this information will be passed on to the Department of Conservation.

The call is a cricket-like trilled creak or whistle - ***Litoria ewingii*** (the Whistling Tree Frog)

6. The call is set of simple harsh croaks - ***Litoria raniformis*** (the Southern Bell frog).

   The call is a prolonged, descending three-syllable drone - ***Litoria aurea*** (the Green and Golden Bell Frog)

7. The frog is in the genus ***Litoria***, use the following questions to determine which species it is.

   Frog has a distinct green or pale stripe down the mid-line of its back - ***Litoria raniformis*** (the Southern Bell frog).

   Frog does not have a distinct line down its back - go to question 8.

8. Frog has pads on the ends of its fingers scarcely wider than digits, it is small (<60 mm), with an overall brown back, usually with a broad dark stripe from the nostril, through the eye to the armpit, and has orange thighs - ***Litoria ewingii*** (the Whistling Tree frog).

   Frog has slightly to poorly developed toe or finger pads, it usually has an overall green coloration with a silver or white stripe or ridge running from eye to groin area and blue thighs. Adults can be quite large (>70 mm) - go to question 9.

9. Frog has a many prominent bumps or warts on its back and very poorly developed toe or finger pads - ***Litoria raniformis*** (the Southern Bell frog).

Frog has a very smooth back, with expanded tips to its fingers and toes which are $1^{1}/_{2}$ times wider than toes or fingers- ***Litoria aurea*** (the Green and Golden Bell Frog).

The Green and Golden Bell Frog - *Litoria aurea*

The Southern Bell frog - *Litoria raniformis*

The Whistling Tree frog - *Litoria ewingii*

Frog Report Form

Date of observation _____

Observer's name and address:

_____

Locality name: _____

Grid reference: _____

Weather: Air temp-_____°C, Cloud -_____, Wind -_____, Rain -_____

Habitat type: _____

Microhabitat description:

_____

_____

_____

Frogs present and reasons for species ID: _____

_____

_____

Description of any deformities noticed: _____

_____

_____

Frogs behaviour: calling - ☐ breeding - ☐ basking - ☐ feeding - ☐

Approx. numbers: _____ other: _____

Frogs present as: adults - ☐ juveniles - ☐ tadpoles - ☐ eggs - ☐

Recording enclosed: yes - ☐ no - ☐ Photo enclosed: yes - ☐ no - ☐

Appendix 2. The New Zealand Frog Distribution Survey in its original format.

New Zealand Frog Distribution Survey

Dr. Stephanie Shaw
New Zealand Centre for Conservation Medicine at Auckland Zoo
Private Bag, Grey Lynn
Auckland
Stephanie.shaw@aucklandcity.govt.nz
Fax 64 9 360-3838; Tel 64 9 353-0752

Did you ever use to find tadpoles when you were a kid? Do you still look? Do you still find them? Have you heard frogs when you were out in the bush? When talking to people about my PhD topic, Amphibian Disease in New Zealand Native Frogs, they often remark on what frogs they are in their backyard or at their favourite forest walk. Although most people actually hear and see the Australian *Litoria* species of frogs, it's still valuable to have the information of where frogs have been and currently are distributed. Dr. Phil Bishop from the University of Otago and a few other collaborators started this project in 2001. We decided that it was due time to ask around again. The results of this survey will be mapped so that population increases and decreases in any geographical area can be recognized. This may also help direct further targeting of monitoring or disease investigations. For more information about New Zealand frogs go to www.nzfrogs.org.nz

Please fill out completely with print letters. In any case if the answer you have does not match the tick boxes please write in.

Q1:**Name**_____
Q2:Postal address_____
Q3:**Email**_____
Q4: **Telephone number with area code**_____
Q5: **Location** (please use the 1:50,000 scale National Topographic Map Sheet names - www.linz.govt.nz (i.e. Napier, Goose Bay, Takapuna):_____
Q6: **Amphibian species in area with relative abundance trend (tick all that apply)**

Note: please note- Maud Island Frog and Stephens Island frog have been omitted as not accessible to public

□ *Litoria aurea* (Green and golden bell frog)	□Increase	□Decrease	□ Stay same
□ *Litoria raniformis* (Southern bell frog)	□Increase	□Decrease	□ Stay same
□ *Litoria ewingii* (Whistling tree frog)	□Increase	□Decrease	□ Stay same
□ *Leiopelma archeyi* (Archey's frog	□Increase	□Decrease	□ Stay same
□ *Leiopelma hochstetteri* (Hochstetter's frog)	□Increase	□Decrease	□ Stay same

Q7: **Was this change** □Sudden □Slow
Q8: **Date(s) of observation (Month/Year (s) you have noticed this trend)** _____
Q9: **Weather conditions (tick all that apply)**

- □ Warm
- □ Cold
- □ Wet
- □ Dry

Q10: **Habitat**

- □ Water tank
- □ Pond
- □ Forest
- □ Stream
- □ Other_____

Q11: **Any observations that you think caused a change?** _____
Q12 **Do you agree to being contacted for more information ?** □Yes □No

Chapter 8: The distribution and host range of *Batrachochytrium dendrobatidis* in New Zealand spanning surveys from 1930-2010

Preamble

Chapter Eight is a critical chapter in the second part of the thesis to assist in answering the question "Is the amphibian chytrid a threat to free-ranging native frogs?" This chapter addresses the first step in this process to ascertain the geographical distribution of *Batrachochytrium dendrobatidis* (Bd) and if possible, how prevalent it was. Many skin swab samples had been taken for specific Bd PCR testing from both native and non-native frogs since 1999 when amphibian chytrid was first discovered in New Zealand. However, most were collected opportunistically without a specific question in mind, and many were in storage awaiting funds and/or a plan for testing. Therefore firstly the large numbers of scattered unpublished Bd results were collated. Then gaps in the dataset were identified and filled in by testing swabs already in storage or by further sampling of frogs. This chapter has collated the New Zealand data into a large dataset that can be maintained separately, but also can be amalgamated into the Australian Bd database. This collation will ensure not only that the unpublished data is not lost, but makes it available for further modelling analyses to predict the locations of Bd in New Zealand based on climatic conditions where it is currently found.

This chapter is the original manuscript and is in the format that is ready to be submitted to Ecological Abstracts as a data paper, while the Abstract is a stand-alone piece which we are submitting to Ecology as part of the same submission.

My contribution: 80%. Amanda Haigh, Ben Bell, Lisa Daglish, Phil Bishop, Rick Speare, Sabine Melzer, Michel Ohmer, Sarah Herbert and I all contributed Bd swabs and/or frog skin for testing and/or results that had previously been unpublished. Virginia Moreno and Ben Bell also supplied Bd data that had been previously published but provided specific location data and lab results for verification for the database. The specifics are listed in the database (Supplementary Material 1). Rachel Summers assisted by creating the GIS map of the data (Appendix 1). Dianne Gleeson assisted by doing some of the Bd-PCR at no cost. Lucy Rowe assisted by identifying frogs for sampling and

arranging permits for samples to be taken at the Otago Museum. Lee Skerratt, Rick Speare, Amanda Haigh, Ben Bell all assisted with the editing of the manuscript. Lee Skerratt assisted me with the review of Bd data, advising new data collection, epidemiology and statistical interpretation of results. The majority of the swabs tested by PCR that came out of my project funds were tested by Stephen Garland at the JCU parasitology laboratory while a few non-native specimens were tested at the Landcare Auckland PCR laboratory. Histology samples were processed at Gribbles Veterinary Laboratories in Auckland and I reviewed all histology slides under the supervision of Lee Berger. I processed all immunoperoxidase (IPX) slides at the JCU histology lab under the supervision of Rebecca Webb and reviewed all IPX results under the supervision of Lee Berger.

The distribution and host range of *Batrachochytrium dendrobatidis* in New Zealand spanning surveys from 1930-2010

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ABSTRACT: Chytridiomycosis caused by the fungal invasive pathogen *Batrachochytrium dendrobatidis* (Bd) was first detected in New Zealand in the Australian introduced frog species *Litoria raniformis* in 1999 in Christchurch. This is still the earliest record and suggests recent introduction into New Zealand. It was detected in the critically endangered *Leiopelma archeyi* in 2001 on the Coromandel Peninsula and has been suggested as responsible for a mass decline (88%) in that population between 1994-2002. We report the current distribution, host species and prevalence where known of the amphibian chytrid fungus *Batrachochytrium dendrobatidis* in New Zealand which is essential for conservation management of New Zealand native frogs (*Leiopelma* spp.). The data set is structured so that it can be readily added to the Australian Bd database to be used for further analyses. Our data included all regions in mainland New Zealand and six off shore islands at 135 sites with 704

records from over eleven contributors spanning collection dates 1930-2010. We report 54 positive sites from 132 positive individuals. We also detail negative findings, but freedom from disease in a location should take into account the sensitivity of the test used and numbers of individuals tested. Included in the data is a comprehensive museum survey which was undertaken testing 152 individuals from five species from 1930-1999 using histology and Bd specific immunohistochemistry. The oldest museum record tested for Bd was from an *L. archeyi* from the Coromandel in 1930. All museum specimens were negative. In *L. archeyi* at a study site in the Coromandel Ranges, the prevalence of Bd from 2006-2010 was relatively stable at 14-18% but testing numbers remain low (up to 18) due to the now low population numbers. In *L. archeyi* in the Whareorino forest, chytridiomycosis was first detected on northern mark recapture monitoring grids in March 2006 at a prevalence of 5% (5/100). The prevalence of Bd in Whareorino has remained both consistent and low (< 50% for the 95% confidence interval upper limit) between 2005-2010. In *L. hochstetteri*, *L. hamiltoni* and *L. pakeka* all sampling for Bd has been negative. Positive Bd results have been found in all three *Litoria* spp. in five out of sixteen regions but Bd has not been found in the six off-shore areas tested). Most of the data has been previously unpublished and represents the first confirmed reports of Bd in many regions and species.

KEY WORDS: amphibian chytrid fungus ·*Batrachochytrium dendrobatidis* · chytridiomycosis · frog · infectious disease · *Leiopelma* · *Litoria* · mapping · New Zealand

INTRODUCTION

Infection with the highly transmissible chytrid fungus *Batrachochytrium dendrobatidis* (Bd) causes the disease chytridiomycosis (Berger et al., 1998; Longcore et al., 1999). Wouldwide, it has caused the decline of around 200 species of frogs (Skerratt et al., 2007) and the greatest loss of vertebrate biodiversity due to disease in recorded history (Berger et al., 1998; Daszak et al., 2000; Skerratt et al., 2007).

New Zealand has four species of extant leiopelmatid native frogs which are primitive frogs with many unique characteristics and are closest related to the tailed frog (*Ascaphus truei*) (Bell and

Wassersug, 2003). All *Leiopelma* are listed by IUCN with the following threat classifications: *Leiopelma archeyi* (critically endangered), *Leiopelma hamiltoni* (endangered), *Leiopelma hochstetteri* (vulnerable) and *Leiopelma pakeka* (vulnerable) (IUCN, 2011). Leiopelmatidae are all nocturnal terrestrial frogs while *L. hochstetteri* is a semi-aquatic stream-dweller (Bell, 1978; Beauchamp et al., 2010). They are all direct developers with the female laying a small clutch of eggs on land and the male guarding these eggs (Bell, 1978). New Zealand also has three species of introduced hylid tree frogs from Australia with the following IUCN threat classifications: *Litoria aurea* (vulnerable), *Litoria ewingii* (least concern) and *Litoria raniformis* (endangered). They are all semi-aquatic with a tadpole phase (Pyke and White, 2001). In New Zealand *Litoria* spp. are only offered limited legislative protection under both the New Wildlife Act 1953 and the Conservation Act of 1987 as they are introduced species (Bishop, 2008).

Most of New Zealand is considered excellent habitat for Bd as it is wet (most areas receive 600-1600 mm of rainfall throughout the year) and mainly temperate with the mean daily minimum temperature (from 1951-1980) ranging from 2.7˚C-11.6˚C and the mean daily maximum temperature ranging from 11.2˚C- 22.0˚C (Leathwick et al., 2002). Bd is pathogenic and virulent over a range of temperatures but has its greatest virulence at ambient temperatures ranging from 12-23˚C (Berger et al., 2004).

In New Zealand, the index case of chytridiomycosis was in December of 1999 of the South Island at Godley Heads, Christchurch in *L. raniformis* (Waldman et al., 2001). Anecdotal reports from many land users in New Zealand report sharp declines in *Litoria* spp. populations all over New Zealand from 1992-1997 (S.Shaw, unpubl. data). A comprehensive museum survey in New Zealand was undertaken testing 152 individuals from 5 species from 1930-1999 using histology and Bd specific immunohistochemistry (Berger et al., 2002). The oldest sample tested was from an *L. archeyi* from the Coromandel in 1930. All museum samples tested negative. Therefore, the earliest record of chytridiomycosis in New Zealand is still 1999 in *L. raniformis* in Christchurch and suggests recent introduction.

The first documented case of chytridiomycosis in native frogs was in the Coromandel population of *L. archeyi* in 2001 (Bell et al., 2004). This appearance of Bd in the population is later than their first population decrease in 1996 (Bell et al., 2004). As discussed in Bell (2004), the causal relationship in the decline and chytridiomycosis has not been proven but extrapolated from many proven cases worldwide (Berger et al., 1998; Lips et al., 2006; Vredenburg et al., 2010).

Since 2004 evidence on the relationship between Bd and the native leiopelmatid frogs has accumulated. Laboratory infection experiments infecting *L. archeyi* (Shaw et al., 2010), *L. hochstetteri* and *L. pakeka* with Bd (Ohmer, 2011) have shown that both *L. archeyi* and *L. pakeka* are susceptible to infection, but self-cure and do not develop clinical chytridiomycosis. It is still unclear if *L. hochstetteri* are able to be infected (Ohmer, 2011). In the wild, *L. hochstetteri* remain negative, despite some of the populations being sympatric with infected *L. archeyi* and *L. aurea* (Bell et al., 2004; S.Shaw, unpubl. data). Both isolated island populations of *L. pakeka* and *L. hamiltoni* have also tested negative (P.Bishop, unpubl. data; S.Shaw, unpubl. data) with *L. pakeka* showing an increasing population since 1983 (Bell, 1994; Bell and Pledger, 2010).

It is still unclear how apparent immunity in *Leiopelma* spp. in the laboratory relates to the 1996 population crash of the Coromandel *L. archeyi*. One scenario is that *L. archeyi* are resistant to clinical chytridiomycosis and the decline in the Coromandel was secondary to a yet undiscovered cause. An alternate hypothesis would be that naïve *L. archeyi* are moderately susceptible to chytridiomycosis in the wild and the disease caused the decline of the Coromandel population. Selection for host resistance and/or reduced pathogen virulence is also possible. In this latter scenario it is also possible that the population effects from chytridiomycosis in the Whareorino population of *L. archeyi* went unnoticed because the population was not monitored prior to 2005. The prevalence of Bd in the mark recapture grids at Whareorino has remained both consistent and low (< 50% for the 95% confidence interval upper limit) between 2005-2010 (L.Daglish, unpubl. data) with no significant difference between the years (S.Shaw, unpubl. data). This stable low prevalence suggests the disease is endemic and may have been introduced before 2005, although this paper reports the first verified report of Bd in the Whareorino. Low prevalence is also consistent with low impact of the disease

(Murray et al., 2009). Further investigation of the mark recapture data as per Murray et al (2009) at Whareorino may help to clarify if there are any seasonal fluctuations as is usually seen and/or impacts on individuals (Berger et al., 2004; Kriger and Hero, 2007; Murray et al., 2009) . In the Coromandel *L. archeyi* population, the prevalence of Bd from 2006-2010 has also been low (B.Bell, unpubl. data).

Scenarios to explain the current situation in apparently resistant and stable populations of *L. pakeka* could be again that previous declines in the wild were not detected. This is unlikely as long term monitoring data from 1983 shows an increasing population (Bell and Pledger, 2010). Therefore *L. pakeka* are either still naïve and could be impacted by the introduction of Bd to isolated populations, or they have been previously exposed but have self-cured and previous infection has not been detected. Either scenario is possible.

In *L. hochstetteri*, both laboratory experiments and sampling from the wild show a resistant, stable population (Baber et al., 2006; Moreno et al., 2011; Ohmer, 2011). It is possible though that again, a decline occurred before monitoring took place and laboratory experiments were done on previously exposed immune individuals.

Chytridiomycosis is also considered endemic in *Litoria* spp., but little is known about exact distribution data of both the frogs and the disease in these introduced species. Seasonal population crashes have been confirmed in both *L. aurea* and *L. raniformis* in the spring months of 2010 (S.Shaw, unpubl. data). Positive Bd results have been found in all three *Litoria* spp. throughout the North and South Islands of New Zealand but Bd has not been found in the three off-shore islands where *Litoria* spp. were tested (Chatham Island, Mayor Island and Ward Island) (P.Bishop and R.Speare, unpubl. data; S.Shaw unpubl. data).

All of the data reported except where referenced are the first published verifiable reports of Bd in New Zealand. Table 1 presents a full list (referenced) of known infected amphibian species in New Zealand following the compilation of the presented data. The current data represents the assemblage of all available and verifiable data on the occurrence of Bd in New Zealand as of 2010. The metadata is modelled after the Australian database so the New Zealand data can be easily amalgamated into one

Australia-New Zealand database (Murray et al., 2010). This work is the result of many contributors who have been collecting frogs and samples for almost 60 years. This is the first comprehensive nationwide database to be compiled and made publicly available to date. The database is updatable and can be used in a both a New Zealand national and global context for predictive modelling, meta-analyses and risk assessment for the management of this devastating, globally invasive disease.

Table 1: Free-ranging amphibian species present in New Zealand recorded as being infected with *Batrachochytrium dendrobatidis* (N=4).

Family	Genus	Species	Reference
Leiopelmatidae	*Leiopelma*	*archeyi*	Bell (2004)
Hylidae	*Litoria*	*aurea* (introduced)	S.Shaw unpubl. data
Hylidae	*Litoria*	*ewingii* (introduced)	S.Shaw unpubl. data, Ohmer (2011)
Hylidae	*Litoria*	*raniformis* (introduced)	Waldman (2001)

Metadata

METADATA

CLASS I. DATA SET DESCRIPTORS

A. Data set identity: The distribution and host range of the invasive disease chytridiomycosis in New Zealand 1930-2010 (Figure 1).

B. Data set identification code: NZ Bd data 1930-2010 (Supplementary Material 1)

Principal Investigators:

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L. F. Skerratt, Amphibian Disease Ecology Group, School of Public Health, Tropical Medicine and Rehabilitation Sciences, James Cook University, Townsville, Queensland 4811, Australia.

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R. Speare, Amphibian Disease Ecology Group, School of Public Health, Tropical Medicine and Rehabilitation Sciences, James Cook University, Townsville, Queensland 4811, Australia.

CLASS II. RESEARCH ORIGIN DESCRIPTORS

A. Overall project description

Identity: The distribution and host range of the invasive disease chytridiomycosis in New Zealand 1930-2010.

Originator: S. D. Shaw, Amphibian Disease Ecology Group, School of Public Health, Tropical Medicine and Rehabilitation Sciences, James Cook University, Townsville, Queensland 4811.

Period of Study: 1930 – 2010.

Objectives: To establish the distribution of amphibian chytridiomycosis in New Zealand.

Abstract: Same as above.

Sources of funding: Auckland Zoo Charitable Trust, Landcare Research Auckland, New Zealand Department of Conservation, New Zealand Frog, New Zealand Royal Forest and Bird Protection Society National Branch, New Zealand Royal Forest and Bird Protection Society Waikato Branch Valder Grant, New Zealand Wildlife Society Marion Cunningham Grant, Wildlife Disease Association Australasian Section. Also study specific – see references.

B. Specific subproject description

Site description: The dataset comprises 135 unique sites in New Zealand that vary in their environmental characteristics. See Land Environment New Zealand database at http://www.landcareresearch.co.nz/databases/LENZ/.

Site type: N/A

Geography: New Zealand: Three distinct islands (North, South and Stewart Islands) north bounding latitude -34.4°; south bounding latitude -44°; west bounding longitude 166.5°; east bounding longitude 178.5° and also six outer islands are included in this study (Chatham Island, Great Barrier Island, Mayor Island, Maud Island, Stephens Island and Ward Island). They are completely surrounded by the Pacific Ocean, the Tasman Sea and a number of straits. The land mass of the study areas is 269,923 km^2 in area. Highest elevation is 3754 m above sea level (a.s.l) (Mt. Cook).

Habitat: Sites represent a diverse range of habitat types across New Zealand, from temperate rainforests to arid and semi-arid lands.

Geology: Various.

Watersheds/hydrology: N/A.

Site history: N/A

Climate: New Zealand has a diverse range of climates: warm subtropical in the far North; high rainfall, temperate regions in the north island and the west coast of the south island; an arid region in the central North Island; semi-arid regions in the interior and east coast of the South Island; and severe alpine conditions in the mountainous areas. Study sites were distributed across all climate zones.

Experimental design: Opportunistic collection of sick and dead amphibians (including apparently healthy museum collections). Details of opportunistic collection can be found in Berger et al.(1998), Berger (2001), Berger et al.(2004) and Weldon et al.(2004). Systematic sampling of healthy amphibians occurred using a number of methodologies but was characterized by replicated sampling of apparently healthy frogs at study sites. Examples of the methodologies typically used for systematic sampling can be found in Baber et al. (2006), Bell (1994), Bell et al.(2004), Bell and Pledger (2010), Haigh et al.(2007), Moreno et al.(2011), Skerratt et al.(2008), Skerratt et al.(2011) and Waldman et al. (2001).

Design characteristics: N/A.

Sampling methods: The diagnostic tests used were histology of skin samples and quantitative PCR of skin swabs, which have estimated sensitivities for wild amphibians of approximately 26.5% and 72.9%, respectively and specificities of 99.5% and 94.2% respectively (Skerratt et al., 2011). Histology testing consisted of serial testing using H & E staining as a screening test and a Bd- specific immunoperoxidase (IPX) test on any suspicious positive or indeterminate samples which improved the sensitivity of histology by 46% (Skerratt et al., 2011). The accuracy of the tests depends on the methodology used in the diagnostic laboratory, which varied during the study. Therefore, single positive records from a location or species must be interpreted with caution. Similarly, establishing freedom from disease in a location or species should include taking the sensitivity of the diagnostic test into account (Skerratt et al., 2008; Skerratt et al., 2011). Details of histological sampling method can be found in Berger et al.(2000). Details of the immunoperoxidase test can be found in Berger et al.(2002). Details of the PCR sampling method can be found in Hyatt et al.(2007).

Taxonomy and systematics: *Batrachochytrium dendrobatidis* (Longcore et al., 1999).

Permit history: New Zealand Department of Conservation low-impact research and collection permits WK-22070-RES, WK- 20068 –RES, NM- 19892- RES, and multiple Otago University Animal Ethics Permits were obtained to test samples collected by the Department of Conservation and for sampling and testing of dead, sick or healthy wild-caught amphibians. Details of the other ethics and collection permits were study specific and can be found within the references provided.

Legal/organizational requirements: None.

Project personnel: The authors.

CLASS III. DATA SET STATUS AND ACCESSIBILITY

A. Status

Latest update: The data represent specimen records spanning collection dates from 1930-2010. Data collection is ongoing and the database will be updated as collected and verified.

Latest Archive date: 10 Feb 2010.

Metadata status: The metadata are complete and up to date.

Data verification: Entries in the database were checked for outliers. Suspicious entries were then re-checked by referral to the relevant original contributors, who are the repositories of original hand-written data.

B. Accessibility

Storage location and medium: (Ecological Society of America data archives [http://esapubs.org/archive/default.htm], URL published in each issue of its journals). Original data are in the possession of individual contributors. Compiled data files are stored on the authors' computers and backup external hard-drives.

Contact person: Lee Skerratt, email: lee.skerratt@jcu.edu.au. Ph (W) +61 7 4781 6065. Fax +61 7 4781 5254. Amphibian Disease Ecology Group, School of Public Health, Tropical Medicine and Rehabilitation Sciences, James Cook University, Townsville, Queensland 4811, Australia.

Copyright restrictions: None.

Proprietary restrictions: None.

Costs: None.

CLASS IV. DATA STRUCTURAL DESCRIPTORS

A. Data Set File

Identity: NZ_Bd_data_1930-2010.txt

Size: 704 records, not including header row.

Format and storage mode: ASCII text, tab delimited. No compression scheme was used.

Header information: See variable names in Section B.

Alphanumeric attributes: Mixed.

Special characters/fields: N/A

Authentication procedures: Sums of the numeric columns are used for cross-checking successful downloads of data file. Year = 1352142, #individuals = 2499, #positive = 132, Latitude = -26445.517, Longitude = 122954.2547.

B. Variable information (Table 2):

CompiledBy: Gives the name of the person responsible for compilation of the data into the database.

DatabaseID: Unique numeric identifier for each row entry.

Species: Gives the species of the specimen that was examined, if available.

Sex: Gives the sex of the specimen examined, if available.

Site: Gives the name or description of the site at which the specimen was collected, if available.

Region: Gives the region in which the specimen was collected, if available.

Country: Gives the country in which the specimen was collected.

Year: Gives the year the specimen was collected, if available.

Diagnostic: Gives the diagnostic method used on the specimen for the detection of *B. dendrobatidis*, if available.

 # individuals: Gives the number of individual frogs examined for each record, if available.

 # individuals positive: Gives the number of individual frogs testing positive for infection with *B. dendrobatidis* from the #individuals examined, if available.

Collector/source: Gives the person/party responsible for the collection and/or submission of the specimen for diagnostic testing, if available.

OR Database: Gives the name of the original database/contact person from which the record was compiled, if available.

Disease Status: Gives the disease status of the record as per the results of diagnostic testing, if available.

Accuracy: Have not used this category but it exists in the Australian database that this data will amalgamate with.

Latitude: Gives latitude of the sites where the specimen was collected (decimal degreesWGS84).

Longitude: Gives longitude of the sites where the specimen was collected (decimal degreesWGS84).

Dead or sick: Provides reference as to whether the specimen was noted as being dead or apparently unhealthy.

Numeric variables: Variables are counts or values of latitude/longitude.

Date variables: Year is supplied.

Table 2: Summary of variable information.

Variable Name	Variable definition	Units	Storage type	Range	Missing value codes
CompiledBy	See above	N/A	Character	N/A	N/A
DatabaseID	See above	N/A	Integer	1 - 704	-9999
Species	See above	N/A	Character	N/A	N/A
Sex	See above	N/A	Character	N/A	N/A
Site	See above	N/A	Character	N/A	N/A
Region Extract	See above	N/A	Character	N/A	N/A
Country	See above	N/A	Character	N/A	N/A
Year	See above	Years AD	Integer	1930-2010	-9999
Diagnostic	See above	N/A	Character	N/A	N/A
#individuals	See above	Count of	Integer	1 - 100	-9999
#positive	See above	Count of	Integer	0 - 14	-9999
Collector/ source	See above	N/A	Character	N/A	N/A
ORDatabase	See above	N/A	Character	N/A	N/A
Disease status	See above	N/A	Character	N/A	N/A
Accuracy	See above	N/A	Character	N/A	N/A
Latitude	See above	Decimal degrees (WGS84)	Floating point	-35.117330 to -46.382490	-9999
Longitude	See above	Decimal degrees (WGS84)	Floating point	167.991005 to 178.369200	-9999
Dead or sick	See above	N/A	Character	N/A	N/A
Notes	See above	N/A	Character	N/A	N/A

CLASS V. SUPPLEMENTAL DESCRIPTORS

A. Data acquisition

Data forms: Various

Location of completed data forms: Various.

Data entry/verification procedures: See earlier comments on data entry and verification (Class III, Section A).

B. Quality assurance/quality control procedures: See earlier comments on data entry and verification (Class III, Section A).

C. Related material: N/A.

D. Computer programs and data processing algorithms: N/A.

E. Archiving: N/A

F. Publications using the data set: None

G. Publications using the same sites: (Bell et al., 2004; Moreno et al., 2011; Waldman et al., 2001)

H. History of data set usage

Data request history: N/A

Data set update history: N/A

Review history: N/A

Questions and comments from secondary users: N/A

Acknowledgments: We thank the following contributors to this database either through assistance in collection of samples or diagnostic testing: Maurice Alley, Peter Anderson, Matt Baber, Lee Berger, Vicki Carruthers, Scott Carver, Robert Chappell, Michael Crossland, Kim Delaney, Stephen Garland, Paul Gasson, Peter Gaze, Jen Germano, Richard Gill, Diana Mendez, Kris Murray, Richard Norman, Oliver Overdyk, Gillian Stone, Mana Stratton, Bruce Waldman, Jess Wallace, Rebecca Webb and Berend Westera.

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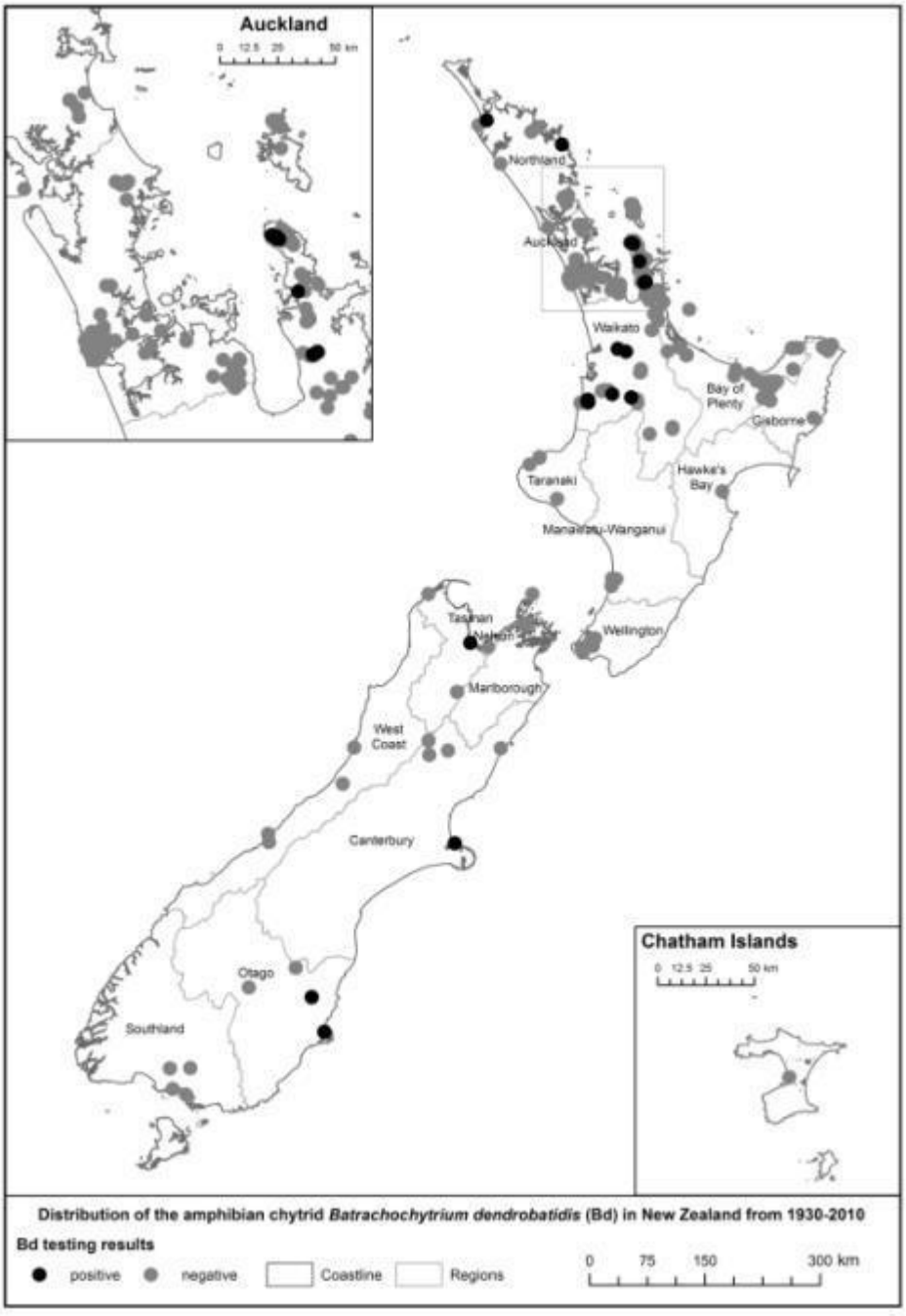
Figure 1: Map of New Zealand showing the distribution of positive sites (black dots; N=54) and negative sites (open squares; N=81) for *Batrachochytrium dendrobatidis* records.



Chapter 9: Experimental infection of self-cured *Leiopelma archeyi* with the amphibian chytrid, *Batrachochytrium dendrobatidis*

Introduction

One of the most important research questions concerning free-ranging New Zealand native frog populations in 2005, was were they at risk for a population crash due to amphibian chytrid, or in the case of the *L. archeyi* Coromandel population, further population crashes? Chapter Nine is a crucial laboratory experiment in this thesis as it tested the susceptibility of *L. archeyi* to chytridiomycosis.

When I started this chapter, *Batrachochytrium dendrobatidis* (Bd) had been found in both wild populations of *L. archeyi* and my surveys were underway. Standardised Bd surveys had not yet been done for the other species but were planned in conjunction with my PhD research. The New Zealand Department of Conservation (DOC) wanted evidence on the risk of the amphibian chytrid to *L. archeyi* populations so that they could plan and budget further surveys, translocations, and prioritize captive breeding. Protocols for captive husbandry, as previously discussed, were also designed around the unknown threat of amphibian chytrid to these insurance populations. The threat to the population of *L. archeyi* at Whareorino was assumed to be the same as the Coromandel population and a large population crash due to chytridiomycosis was predicted. To avoid this, DOC captured and tested 100 *L. archeyi* for a translocation to a new patch of forest with minimal human and introduced frog contact, as another way to provide for a wild insurance population. Of the 100 frogs, 12 tested positive during the 90 day quarantine and transferred to the University of Otago for further research. However, when retested, these frogs were all negative by PCR for Bd and remained so, as well as remaining healthy (Bishop et al., 2009). This breakthrough reshaped the entire way of thinking about *Leiopelma* spp. and the amphibian chytrid. If *L. archeyi* could self-cure when infected naturally in the wild, were they really at risk? If not, why did the *L. archeyi* Coromandel population appear to crash from chytridiomycosis? One of the first keys to answer to assess the risk of amphibian chytrid to the wild frogs was could the self-cured frogs be reinfected and would they self-cure again? This question also applied to the other *Leiopelma* spp., but due to time constraints my goal was to address the issue in *L. archeyi* Whareorino population as twelve wild Archey's frogs became available for this research.

This chapter is the original manuscript as published in a peer-reviewed journal: Shaw SD, Bishop PJ, Berger L, Skerratt LF, Garland S, Gleeson DM, Haigh A, Herbert S, Speare R (2010). Experimental infection of self-cured *Leiopelma archeyi* with the amphibian chytrid *Batrachochytrium dendrobatidis*. Diseases of Aquatic Organisms 92:159-163.

My contribution: 85% (detailed in co-author publication release form at the end of this chapter).

Vol. 92: 159–163, 2010
doi: 10.3354/dao02227

DISEASES OF AQUATIC ORGANISMS
Dis Aquat Org

Published online May 26, 2010

Contribution to DAO Special 4 'Chytridiomycosis: an emerging disease'

Experimental infection of self-cured *Leiopelma archeyi* with the amphibian chytrid *Batrachochytrium dendrobatidis*

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ABSTRACT: The susceptibility of Archey's frog *Leiopelma archeyi* to *Batrachochytrium dendrobatidis* (*Bd*) is unknown, although one large population is thought to have declined sharply due to chytridiomycosis. As primary infection experiments were not permitted in this endangered New Zealand species, 6 wild-caught *L. archeyi* that naturally cleared infections with *Bd* while in captivity were exposed again to *Bd* to assess their immunity. These frogs were from an infected population at Whareorino, which has no known declines. All 6 *L. archeyi* became reinfected at low intensities, but rapidly self cured, most by 2 wk. Six *Litoria ewingii* were used as positive controls and developed heavier infections and clinical signs by 3 wk, demonstrating that the zoospore inoculum was virulent. Six negative controls of each species remained uninfected and healthy. Our results show that *L. archeyi* that have self cured have resistance to chytridiomycosis when exposed. The pattern is consistent with innate or acquired immunity to *Bd*, and immunological studies are needed to confirm this.

KEY WORDS: *Leiopelma archeyi* · *Litoria ewingii* · *Batrachochytrium dendrobatidis* · Chytridiomycosis

INTRODUCTION

New Zealand has 7 extant species of frogs. The 4 native species are *Leiopelma archeyi*, *L. hamiltoni*, *L. hochstetteri* and *L. pakeka*. Leiopelmatids are an archaic family of anurans with many unique features such as vestigial tail-wagging muscles, cartilaginous inscriptional ribs, the presence of amphicoelous vertebrae and 9 presacral vertebrae (Bishop et al. 2008). They are all direct developers; *L. hochstetteri* is nidicolous while the others are exoviviparous with the hatchlings completing development on the male's dorsum (Bell & Wassersug 2003). All 4 species are on the top 60 Evolutionarily Distinct and Globally Endangered (EDGE) amphibian list, with *L. archeyi* in the No. 1 position (www.edgeofexistence.org). In addition, all are nationally threatened, with *L. archeyi* classified as endangered (Hitchmough et al. 2005). *L. archeyi* has 2 geographically distinct populations; *L. hamiltoni* and *L. pakeka* have just one naturally occurring population each and *L. hochstetteri* has over 10 distinct populations (Bishop et al. 2008). *Litoria aurea*, *L. ewingii* and *L. raniformis* were introduced from Australia over 100 yr ago, and in their country of origin all 3 are on the IUCN red list (www.iucnredlist.org).

In 1999, *Batrachochytrium dendrobatidis* (*Bd*) was found in New Zealand in dying *Litoria raniformis* in a pond near Christchurch (Waldman et al. 2001). It is now known to be widespread in New Zealand and has been found in all *Litoria* species and *Leiopelma archeyi* (Bell et al. 2004, Shaw et al. 2008a). Limited testing did not detect infections in *L. hochstetteri*, *L.*

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pakeka (Shaw et al. 2008a) and *L. hamiltoni* (P. Bishop unpubl. data). Chytridiomycosis has spread worldwide, causing massive die-offs leading to amphibian population declines and extinctions (Skerratt et al. 2007, Padgett-Flohr & Hopkins 2009). However, its past and potential impacts on New Zealand's unique amphibians, and its time of arrival, are unknown.

In 2001, chytridiomycosis was discovered in one dead *Leiopelma archeyi* in the Coromandel Peninsula following a sharp population decline in 1995 (Bell et al. 2004). As other potential causes for the declines in *L. archeyi* such as predation, habitat loss and poisons were ruled out, the introduction of *Bd* was suggested as the most likely cause (Bell et al. 2004). In 2005, chytridiomycosis was detected in the only other population of *L. archeyi.* Since this population in the Whareorino forest had not appeared to have declined (although long-term studies had not been conducted), there was concern the disease had just arrived, so an emergency translocation was initiated (Haigh et al. 2007). Twelve of 100 *L. archeyi* captured for the translocation that tested positive for *Bd* during a 90 d quarantine period were transferred to the University of Otago for use in a treatment trial. However, when retested at the research facility, all except one frog had self cured. The last frog also became negative after a short course of topical chloramphenicol (Bishop et al. 2009). In addition to this unexpected self cure in captive frogs held at conditions ideal for *Bd*, the prevalence of *Bd* in the Whareorino population increased over the next 3 yr, but the abundance of frogs did not appear to decline (Shaw et al. 2008a). Therefore, the question arises as to why there was a population decline in *L. archeyi* on the Coromandel peninsula, but not in the Whareorino, as they have a similar habitat, altitude and climate. One hypothesis is that *Bd* arrived at Whareorino before the population was monitored, and the disease is now in a stable endemic phase. Small stable remnant populations after initial large declines have been reported in a few Australian species, such as *Taudactylus eungellensis* (Retallick et al. 2004), and some species have recovered some of their previous distribution and abundance, such as *Litoria genimaculata* (McDonald et al. 2005). This may also have occurred in *L. archeyi* in the Coromandel (Shaw et al. 2008a). An alternate hypothesis is that *L. archeyi* are innately resistant to *Bd* and the population crash in the Coromandel was caused by an undetermined unrelated factor or co-factor that makes *L. archeyi* susceptible to *Bd*.

Little is known about immunity to *Bd* in amphibians, although there is great variability in susceptibility among species and individuals, with some species undergoing no mortality (Berger et al. 2009). Some species of amphibians can clear infection at high temperatures, and there is variable pathogenicity among fungal strains (Berger et al. 2004). Mechanisms of resistance that are under investigation include aspects of innate immunity, such as dermal antimicrobial peptides and resident bacterial flora (Rollins-Smith & Conlon 2005, Woodhams et al. 2006, Harris et al. 2009). The acquired immune response to *Bd* has not been described.

This study aimed to test the following: (1) whether *Leiopelma archeyi* that self cured their naturally acquired infection with *Bd* could be reinfected by experimental challenge with *Bd*; (2) whether reinfected *L. archeyi* developed clinical signs of chytridiomycosis; and (3) whether reinfected *L. archeyi* could again self cure. Information on the susceptibility of *L. archeyi* is clearly needed to determine the threats to, and to plan management of, this critically endangered species.

MATERIALS AND METHODS

The 12 *Leiopelma archeyi* used in this experiment (6 controls, 6 to be exposed) were wild caught in the Whareorino forest in the Waikato Region of New Zealand (ca. 38° S, 174° E) in September 2006 and quarantined for 90 d at Hamilton Zoo (Bishop et al. 2009). All had 3 *Bd* PCR tests done 14 d apart in September and October 2006 (Haigh et al. 2007). They were retested upon arrival at the University of Otago in December 2006 and many times thereafter. Eleven of the 12 frogs had 7 negative tests in a row over 19 wk. The last frog had 6 negative tests in a row, as the first test upon arrival was positive (Bishop et al. 2009). The 12 *Litoria ewingii* used as positive and negative controls were wild-caught from Macraes Flat in the Otago Region of New Zealand (45° 22' S, 170° 25' E) up to 12 mo before the experiment. *L. ewingii* is known to be susceptible to chytridiomycosis (Berger et al. 2004). It is similar in size to *L. archeyi* and hence provides a surface area that is valid for comparison of zoospore equivalents on PCR. All frogs were given a physical examination by a veterinarian and were negative by *Bd* PCR prior to the experiment. Upon entering the study, one *L. archeyi* positive control had nodular skin lesions that were thought to be adenomas (Shaw et al. 2008c). Since these lesions have been seen on numerous *L. archeyi* and are not known to cause morbidity (Shaw & Holzapfel 2008), the frog was retained in the experiment.

Frogs were housed individually in semi-transparent plastic containers (310 × 210 × 90 mm) on 3 paper towels moistened with aged filtered water and changed weekly. The containers were housed on 2 shelving units in an isolated refrigerated room kept at 15°C and

close to 100% humidity. This is similar to the average maximum temperature of the natural habitat of *Leiopelma archeyi*, which is 15.2°C (Eggers 1998). One shelving unit held *L. archeyi* and the other held *Litoria ewingii*, with the positive and negative controls on different shelves. Strict hygiene practices were used to avoid cross-contamination by servicing negative controls first and using new nitrile gloves for each frog. Frogs were weekly fed a varying diet of laboratory-cultured insects consisting of waxmoth larvae *Galleria*, fruit flies *Drosophila*, small black crickets *Teleogryllus commodus* and house flies *Mus musca*.

As a New Zealand isolate of *Bd* was not available, a culture of type isolate JEL 197 (Longcore et al. 1999) was sourced from a cryo-archived culture (Boyle et al. 2003) held at the Department of Biochemistry, University of Otago, by R. Poulter and M. Butler; it had been passaged on 1% tryptone plates at 23°C for about 1 yr prior to this experiment. Zoospores were collected from 4 d old cultures on 1% tryptone agar plates held at 23°C by flooding with 6 ml of dilute salt solution (DSS, imitating pond water; Boyle et al. 2004). The number of live zoospores present in the DSS was determined using a haemocytometer. The 6 *Leiopelma archeyi* to be exposed and the 6 positive control *Litoria ewingii* were each exposed to approximately 250 000 zoospores (within 30 min of zoospore collection) in 10 ml DSS in 100 × 130 mm plastic bags with a ziplock seal for 4 h at 15°C (a temperature suitable for *Bd*). The 12 negative controls had the same treatment but without *Bd* zoospores. The sealed plastic bags prevented the frogs from climbing out of the water. The frogs were then returned to their containers together with the remaining liquid from the plastic bags.

Exposed frogs were swabbed for PCR testing every 7 d for up to 12 wk post exposure. Frogs were observed daily, and at the time of swabbing they were also weighed and examined for clinical signs of *Bd* such as erythema, lethargy, irregular posture, inappetance and neurological signs (Berger et al. 2005). Taqman quantitative PCR (qPCR) for *Bd* (Boyle et al. 2004) was performed on the swabs and run in triplicate for each sample at both James Cook University (JCU) and Landcare Research Ltd laboratories (LR) for the pre-data and Week 1 for all frogs, then the first 4 wk for *Leiopelma archeyi* alone. This double testing was carried out to validate the results obtained at LR, where the assay had been recently established, against those obtained at JCU, a laboratory experienced in running the assay. There were no significant differences in results from both laboratories (data not shown). The additional *L. archeyi* swabs were all tested at JCU while the remaining *Litoria ewingii* swabs were all tested at LR for confirmation of continued infections. Two facilities were used for funding reasons. Results for a sample were only considered positive if all 3 qPCR reactions were positive. Inhibition of the PCR was tested using an Applied Biosystem TaqMan® Exogenous Internal Positive Control (EIPC) (Hyatt et al. 2007).

The *Litoria ewingii* positive controls were transferred to a treatment experiment at Week 4, when they began developing clinical signs of chytridiomycosis and were likely to die within days if left untreated (Berger et al. 1998).

These experiments were conducted under the following permits: animal ethics from the University of Otago (AEC 88/06) and James Cook University (A-1315) and Department of Conservation High Impact permit (WK-19818-RES).

RESULTS

Leiopelma archeyi

At the end of Week 1, all exposed *Leiopelma archeyi* were positive with low zoospore equivalents of 2 to 915 per sample (Table 1). *Bd* was rapidly cleared as demonstrated by only 2 of the 6 exposed frogs testing positive at Week 2, and by Week 3 all PCR results were negative. At Week 4, 1 of the frogs that was positive at Week 2 returned a positive result with a low zoospore equivalent, but all frogs were negative when retested at Weeks 5, 6, 9 and 12. The 6 negative control *L. archeyi* were negative for *Bd* at Week 1 and remained negative.

Exposed frogs either gained or maintained their weight and did not show clinical signs of chytridiomycosis. However, one exposed frog that was negative from Week 2 did show clinical signs of illness (lethargy, decreased righting reflex, tremors) at Week 5. These signs were attributed to metabolic bone disease, as this

Table 1. *Batrachochytrium dendrobatidis* (*Bd*) infecting *Leiopelma archeyi* and *Litoria ewingii*. Percent frogs positive (Pos) for *Bd* after exposure and zoospore equivalents. Each group had 6 frogs. *L. ewingii* were removed from the trial at Week 4 and treated. ZE: zoospore equivalents (mean ± SD of infected frogs)

Time	*Leiopeima archeyi*		*Litoria ewingii*	
	Pos	ZE	Pos	ZE
Day 0	0.00	0	0.00	0
Week 1	100.00	186.7 ± 362.9	83.30	5821.7 ± 4872.5
Week 2	33.30	75.5 ± 102.5	100.00	16544.5 ± 15457.5
Week 3	0.00	0	100.00	8202.7 ± 5608.6
Week 4	16.70	27	100.00	6964.62 ± 6567.2
Week 5	0.00	0	Not tested	

was a problem in another institution holding *Leiopelma archeyi* (Shaw et al. 2008b). Due to the possibility that all *L. archeyi* were calcium deficient, coupled with the fact that these are critically endangered species, treatment with percutaneous calcium/Vitamin D3 preparation (Calcivet®) every 4 to 7 d was initiated in all *L. archeyi* at Week 5. In addition, the sick frog received daily baths of 2.5% calcium gluconate and 7 d of 2% chloramphenicol baths for its antibacterial effects. Hyatt et al. (2007) recommended 3 consecutive negative results over a 14 d period to confirm a negative result. As this frog had been negative for *Bd* for 3 consecutive weeks, it is thought that the chloramphenicol treatment had no bearing on the subsequent negative tests.

Litoria ewingii

At Week 1 post exposure, all 6 exposed *Litoria ewingii* were infected, with zoospore equivalents per sample of 2200 to 14 000 (Table 1). All developed clinical signs of chytridiomycosis (erythema, increased sloughing of skin and lethargy) by Week 3 post exposure, and all frogs were transferred to a treatment experiment at Week 4. The 6 unexposed *L. ewingii* (negative controls) returned negative *Bd* PCR results at Week 1 and remained negative. One frog was euthanised at Week 6 due to accidental trauma, and 1 was treated with an ophthalmic preparation of chloramphenicol due to corneal prolapse at Week 3.

DISCUSSION

Leiopelma archeyi individuals that had self cured from natural infections of *Bd* (Bishop et al. 2009) were exposed to *Bd*, became infected and quickly self cured. Self curing has been reported in 4 free-living *Litoria pearsoniana*, but all at higher temperatures (19.5 to 27°C) than our laboratory experiment (15°C; Murray et al. 2009), which may have aided in their recovery (Berger et al. 2004). The infection of all 6 *L. ewingii* with development of clinical signs demonstrated that the *Bd* isolate was capable of transmission and was virulent. The experimental conditions were validated, and the results support the observation that *L. archeyi* from the Whareorino population are resistant to *Bd* infection. The results also support the possibility that a non-specific innate immune response or a specific acquired immune response influences resistance to *Bd* in *L. archeyi*. Without data on anti-*Bd* antibodies or cellular immune responses to *Bd*, only indirect evidence can be used to indicate whether immunity caused elimination of *Bd*. More rapid clearing at rechallenge than with a primary exposure would support specific acquired immunity, but for these frogs the response to their initial infections with *Bd* is not known. Hence, the mechanism for the rapid elimination of *Bd* from these *L. archeyi* remains to be determined.

Our findings that frogs from Whareorino have resistance to *Bd* supports the field data showing that populations are not declining even though *Bd* is present and prevalence is increasing (Shaw et al. 2008a). However, many free-living *Leiopelma archeyi* have very low zoospore counts (Bishop et al. 2009). This could result in false negative tests in the field and underestimation of prevalence. We hypothesise that longitudinal monitoring of individuals in mark-recapture studies may show a repeating cycle of reinfection and elimination and varying levels of prevalence.

Knowing that *Leiopelma archeyi* from the Whareorino population do not usually become clinically ill and clear infections with *Bd* suggests that a population decline is less likely to be imminent. This information would give the New Zealand Native Frog Recovery Group more options for captive management. Currently all captive populations of *L. archeyi* are maintained indoors with strict hygiene controls aimed at excluding chytridiomycosis (Shaw & Holzapfel 2008). Unsatisfactory breeding and a high prevalence of metabolic bone disease in captivity (Shaw et al. 2008b) have given rise to question the sole use of indoor enclosures. However, if *Bd* is a minimal threat to this species, outdoor enclosures previously thought too risky can be used.

As New Zealand strains of *Bd* had not been isolated, we used an American isolate that could differ in virulence from those present at Whareorino. Isolates from *Litoria ewingii* in New Zealand have recently been cultured and have been sequenced (R. Poulter unpubl. data, S. Shaw unpubl. data), but none have been isolated from *Leiopelma* spp. This experiment should be repeated with isolates from New Zealand and with individuals from the Coromandel population, incorporating assessments of cellular and acquired immune responses.

A population genetic study of *Leiopelma archeyi* should also be undertaken to determine the likelihood of a previous population decline in the Whareorino population. Differences in the levels of resistance between the 2 populations in conjunction with the results of the genetic studies would be used to identify whether factors that influence resistance are intrinsic to the frog or are site specific. A higher genetic diversity in the Whareorino population would support the hypothesis that factors specific to the Coromandel population reduced resistance causing the documented population decline. A subsequent finding of no difference in resistance between the 2 populations would

suggest that factors extrinsic to the frog, such as increased pathogenicity of *Bd*, presence of other pathogens, poisons or other site-specific influences and interactions caused the decline. Lower resistance in the Coromandel population would suggest intrinsic genetic differences that influence immunity or resistance in general. Alternatively, if there was a previous population decline at Whareorino, potentially caused by *Bd*, this would suggest that there are no factors that reduce resistance that are specific to Coromandel, especially if there are no differences in resistance between the 2 populations. A subsequent finding of reduced levels of resistance at Coromandel would again suggest intrinsic genetic differences that influence immunity or resistance in general. The scenarios would require different revisions to management and research plans, revisions that would be urgent if highly pathogenic strains of other pathogens were implicated.

Acknowledgements. We acknowledge a grant to S.D.S. from JCU Graduate Research Scheme. We thank J. Germano and J. Oldman for animal care.

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Editorial responsibility: Alex Hyatt, Geelong, Victoria, Australia

Submitted: August 6, 2009; Accepted: April 12, 2010
Proofs received from author(s): May 20, 2010

Consent of Authors for previous published document.

Chapter #	Details of publication(s) on which chapter is based	Nature and extent of the intellectual input of each author	I confirm the candidate's contribution to this paper and consent to the inclusion of the paper in this thesis	Name (please print):	Signature
NINE	Shaw SD, Bishop PJ, Berger L, Skerratt LF, Garland S, Gleeson DM, Haigh A, Herbert S, Speare R 2010. Experimental infection of self-cured Leiopelma archeyi with the amphibian chytrid Batrochochytrium dendrobatidis. Diseases of Aquatic Organisms 92(2-3): 159-163.	Stephen contributed to the ideas and editing the conclusion in relation to explaining the possible differences between the Whareorino and Coromandel frog populations.	Yes that is correct.	Stephen Garland	
	same	Lee contributed to the paper by thorough editing of the MS.	Yes that is correct.	Lee Berger	
	same	Richard contributed to the paper by thorough editing of the MS, and helping construct the table. He also helped with the formation of the idea of the reinfection experiment.	Yes that is correct.	Richard Speare	
	same	Phil contributed by providing the laboratory, assisting with the experiment, helping obtain NZ permits, and by minor editing of the MS.	Yes that is correct.	Phil Bishop	
	same	Lee contributed to the paper by assisting with testing protocols, interpreting results, and minor editing of the MS.	Yes that is correct.	Lee Skerratt	
	same	Dianne contributed to the paper by providing some of the PCR testing of skin swabs and minor editing of the methods section of the MS.	Yes that is correct.	Dianne Gleeson	
	same	Amanda contributed to the paper by assisting with permits and minor editing of the introduction of the MS.	Yes that is correct.	Amanda Haigh	
	same	Sarah contributed to the paper by assisting with the lab work portion of the reinfection experiment and minor editing of the MS.	Yes that is correct.	Sarah Hirst	

Chapter 10: Baseline cutaneous bacteria of free-living New Zealand native frogs (*Leiopelma archeyi* and *Leiopelma hochstetteri*) and implications for their role in defence against the amphibian chytrid (*Batrachochytrium dendrobatidis*)

Preamble

The aims of this chapter were to:

1) establish baseline bacterial skin flora in free-living native frogs; and

2) test some of these bacterial isolates against a New Zealand isolate of amphibian chytrid to identify any that inhibit the growth of Bd *in vitro*.

This chapter concept originated from two specific issues occurring in Archey's frogs before and during the project. First, there were many bacterial infections reported as a cause of morbidity and mortality in captive frogs, but little data on normal flora in free-living frogs (Potter and Norman, 2006; Shaw and Holzapfel, 2008; Shaw et al., 2012). Second, was the growing evidence that both *Leiopelma archeyi* and *Leiopelma hochstetteri* showed some resistance to chytridiomycosis (Shaw et al., 2009; Shaw et al., 2010). Worldwide the role of antimicrobial peptides and bacteria in innate resistance was being investigated with promising results of finding bacteria with *in vitro* anti-Bd properties (Becker et al., 2012; Harris et al., 2006; Harris et al., 2009; Lam, 2010). Current studies are aimed at using these bacteria as bioaugmentation to improve survival in wild or reintroduced amphibians threatened by chytridiomycosis. (Lips et al., 2005; Skerratt et al., 2007).

Although the Bd - bacteria challenge assay posed technical difficulties, the important finding that bacteria can inhibit Bd implies that cutaneous bacteria may play a role in the innate immunity of *Leiopelma* spp. against Bd.

This chapter is written to be submitted to the Journal of Wildlife Diseases post-thesis with appropriate journal specific changes to the content and format.

My contribution: 90%. The collection of bacterial swabs was done by Amanda Haigh and Lisa Daglish from the Department of Conservation under their own permits. DNA extraction, PCR and DNA sequencing for bacterial identification were performed by Daniel Than from Landcare Research Auckland. The rest of the bacterial identification process was performed by me with assistance from Sara Bell (JCU) and Sarah Dodd (Landcare Research, Auckland). Tim James performed the genetic analysis of the New Zealand Bd isolate and constructed the phylogenetic tree.

Baseline cutaneous bacteria of free-living New Zealand native frogs (*Leiopelma archeyi* and *Leiopelma hochstetteri*) and implications for their role in defence against the amphibian chytrid (*Batrachochytrium dendrobatidis)*

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ABSTRACT: Ninety-two unique bacterial isolates from the ventral skin of sixty-two apparently healthy *Leiopelma archeyi* and *Leiopelma hochstetteri* native frogs from the Coromandel and Whareorino regions in New Zealand were identified using molecular techniques. The most common isolates identified in *L. archeyi* were *Pseudomonas* spp. and the most common in *L. hochstetteri* were *Flavobacterium* spp. Knowledge of baseline cutaneous bacterial flora may be important in interpreting diagnostic cultures from captive sick frogs, quarantine or pre-translocation disease screening. Bacteria may also be an important part of innate immunity in *L. archeyi* and *L. hochstetteri* against chytridiomycosis. A New Zealand strain of *Batrachochytrium dendrobatidis* (Bd) (KVLe08SDS1) was isolated for the first time and used against bacterial isolates in an *in vitro* challenge assay to test for Bd inhibition. One of 21 bacterial isolates tested, a *Flavobacterium* sp., inhibited the growth of Bd. These results imply that cutaneous bacteria may play a role in the innate defence in *Leiopelma* against pathogens, including Bd, and are a starting point for further investigation.

KEY WORDS: amphibian chytrid · bacteria · *Batrachochytrium dendrobatidis* · innate immunity · *Leiopelma archeyi* · *Leiopelma hochstetteri* · quarantine · translocation

INTRODUCTION

New Zealand native frog fauna is comprised of four species of extant Leiopelmatids with the following I.U.C.N. classifications: *Leiopelma archey*i (critically endangered), *Leiopelma*, *hamiltoni* (endangered), *Leiopelma hochstetteri* (vulnerable) and *Leiopelma pakeka* (vulnerable) (IUCN, 2011). They are all also listed in the top 100 amphibian species of the most evolutionarily distinct and globally endangered list with *L. archeyi* holding the top position (E.D.G.E., 2011). All are nocturnal, terrestrial frogs except *L. hochstetteri* which are semi aquatic.

In 1996 one of the two known populations of *L. archeyi* underwent a severe population crash (Bell et al., 2004). The cause of the decline was thought to be from chytridiomycosis, as in many other amphibian populations worldwide (Berger et al., 1998; Daszak et al., 2000; Lips, 1999; Skerratt et al., 2007; Vredenburg et al., 2010). This find sparked the testing of populations of *L. archeyi* in the Whareorino and the approximately 22 (Baber et al., 2006) known populations of *L. hochstetteri* (Shaw et al., 2009) for *Batrachochytrium dendrobatidis* (Bd). The Whareorino population of *L. archeyi* was found to be infected with Bd, but six monthly monitoring since 2005 has shown the population size is stable (Shaw et al., 2009). Monitoring of the *L. hochstetteri* populations has been sporadic but Bd has not been detected and their populations also appear to be stable (Baber et al., 2006; Shaw et al., 2009; Whitaker and Alspach, 1999).

Due to the global amphibian declines, amphibians are frequently brought into captivity and transferred between institutions for captive reproduction and treatment. Currently, routine bacterial skin cultures are not collected as part of quarantine procedures (Pessier and Mendelson, 2010) and consequently there is little data available on the baseline cutaneous bacterial flora in free-living amphibians. Therefore, when skin cultures from sick animals are analysed (Pessier, 2002), it is difficult to tell what organisms are likely to be pathogens and which are part of the normal bacterial microbiota. Bacterial cultures have been performed before from the dorsal skin surface on both captive and free-living *L. archeyi* from both the Coromandel and Whareorino populations (Potter and Norman, 2006). That study identified 41 different bacteria using standard morphological and biochemical tests and found that the bacterial skin flora differed between captive and free-living frogs

and between locations of free-living frogs. However, as the bacterial swabs were taken only from the dorsal skin surface, the results may not be a true indication of the full spectrum of bacterial species present (Culp et al., 2007).

Amphibian species vary in their ability to resist Bd infection and their susceptibility to population declines. For example, in the case of New Zealand frogs, laboratory infection experiments using Bd in *L. archeyi* and *L. pakeka* have shown they are able to be infected, but self-cure rapidly and do not show clinical signs (Ohmer, 2011; Shaw et al., 2010). *In-vitro* experiments in *L. hochstetteri* have shown equivocal results and indicate they are likely resistant to infection (Ohmer, 2011). Adaptive (acquired) immunity has not been found to play a role in Bd defence (Rosenblum et al., 2009; Stice and Briggs, 2010) until recently where one study demonstrated that the typically Bd-resistant African clawed-frog (*Xenopus laevis*) showed both an adaptive and innate immune response (Ramsey et al., 2010).

Many factors can contribute to host vulnerability, such as Bd strain, temperature and season (Berger et al., 2004; Berger et al., 2005). However, innate skin defences such as antimicrobial peptides are thought to play a major role in preventing skin infection by Bd (Ramsey et al., 2010; Rollins-Smith et al., 2006; Rollins-Smith, 2009; Woodhams et al., 2005; Woodhams et al., 2006). Experiments with skin peptides of *L. archeyi*, *L. hochstetteri* and *L. pakeka* have shown that *L. archeyi* skin peptides have the highest *in vitro* activity against Bd and may play a vital role in their initial defence (Melzer and Bishop, 2010). Another aspect of innate defence is the cutaneous bacterial flora, and many bacterial species produce metabolites that inhibit growth of Bd on nutrient agar (Harris et al 2009). It has been shown that, in some frog species, individuals with inhibitory bacteria are able to resist Bd, while those individuals without these beneficial bacteria succumb (Becker et al., 2012; Harris et al., 2009). Using probiotic symbiotic bacteria as a treatment to protect amphibians against chytridiomycosis has had mixed success (Becker et al., 2012; Harris et al., 2009; Woodhams et al., 2011).

The objectives for the study were twofold: 1) To obtain baseline cutaneous bacterial flora data from the ventral skin of *L. archeyi* and *L. hochstetteri* and 2) To test the bacteria against a New Zealand isolate of Bd in vitro to see if bacterial metabolites were produced that could prevent Bd growth. We hoped to gain insight into the apparent immunity to Bd in leiopelmatid frogs and aid further development of bacteria as a bioaugmentation tool in amphibian species susceptible to chytridiomycosis.

MATERIALS AND METHODS

Sample Collection for Cutaneous Bacteria

In February 2009, The New Zealand Department of Conservation staff collected swab samples from 33 *L. archeyi* and 20 *L. hochstetteri* in the Whareorino forest (-38.4, 174.8) and 11 *L. archeyi* from the Coromandel Peninsula (-36.5, 175.4) of New Zealand. The ventral surface of all frogs was washed twice with either sterile water (10 ml plastic vials; Astra Zeneca Ltd., North Ryde, Australia) in the Coromandel, or rainwater, in the Whareorino, to remove surface dirt. The frogs were then swabbed to collect skin bacteria using a sterile transport swab. This was placed into sterile collection media (Copan, Via F., Perotti, Brecia, Italy), transported to the lab in a chilled container and plated on nutrient agar within 48 hours of collection.

Bacterial Culture and Identification

Bacteria were transferred from the swabs onto TGhL agar plates (Longcore et al., 1999) within a laminar flow cabinet at Landcare Research (Auckland, New Zealand). Swabs were wiped over the surface of the agar in the plate whilst rotating the tip of the swab to ensure complete transfer. Agar plates were incubated in the dark at 18°C to simulate normal growth conditions of the ventral surface of *L. archeyi*. Plates were checked daily and obvious single colonies of bacteria were transferred to a fresh agar plate and isolated to pure culture. Each pure culture was given a unique identification number and stored on TGhL agar slants at 4°C. Pure cultures were compared and, for each frog species and site, those bacteria that had similar morphology were grouped together. Given

the projects financial constraints only one representative from each of the morphologically distinct groups was subsequently identified by 16S rRNA sequencing (Landcare Research, Auckland, New Zealand). DNA was extracted using a Sigma REDExtract-N-Amp™ Tissue kit following the manufacturer's instructions (Sigma-Aldrich, Castle Hill, New South Wales, Australia). The extracted DNA samples were then amplified using the bacterial 16S rRNA primers 1F and 1509R (Normand 1995) and the following PCR conditions; 95°C for 4 min; 95°C for 30 s, 53°C for 30 s, and 72°C for 1 min for 25 cycles; and 72°C for 10 min. Successful amplifications were then confirmed by running the PCR products on a 1.5% (wt/vol) agarose gel at 150V for 30 minutes, staining with ethidium bromide and visualising under UV light. The PCR products were then sequenced using an ABI Genetic Analyser 3130xl sequencing machine (Applied Biosystems, Mulgrave, Victoria, Australia). The resulting sequence data were analysed using the Sequencher software v. 5.0 (http://genecodes.com), and identities confirmed by BLAST search (NCBI ref) using the program Geneious (v.5.65) (Drummond et al., 2012).

To assess if location and/or species affected the presence or frequency of bacterial genera identified, the data were analyzed using Fisher's Exact Tests with WINPEPI statistical programme (v. 11.20) (Abramson, 2011).

***In vitro* Bacterial Challenge Assay**

Thirty-one bacterial isolates from the Coromandel population of *L. archeyi* were challenged against Bd using the technique described by Harris et al (2006). All procedures were performed using sterile methods in a class 2 biosafety cabinet. A NZ isolate of Bd was cultured by standard methods (Berger et al 2005) and identified as a unique genotype (Appendix 1). Actively growing Bd cultures in TGhL broth were passaged to TGhL agar plates (Berger et al., 2009) and incubated at 15°C. After three days, zoospores were collected by flushing plates with six ml sterile distilled water. Zoospores were counted using a Neubauer hemocytometer and resuspended to a concentration of 4,000,000 zoospores/ml. One ml of the zoospore suspension was spread evenly on a new TGhL plate and air-dried in a sterile biohazard cabinet until the plate appeared dry but still glistening. Then one streak of each freshly cultured identified bacterium was made on the left side of the plate and a sterile loop with

no bacteria was used to make a streak on the other side of the plate as a negative control. This process was repeated until a bacterium that caused no inhibition of Bd was found (*Chryseobacterium sp. 3A blue*). From then on this was used as a negative bacterial control on the right side of the plate, in place of the sterile streak.

The plates were inspected 24, 48, and 72 hours after inoculation and scored in one of three ways: 1) positive inhibition if there was Bd growth and a zone of inhibition around the bacterial streak; 2) negative inhibition if there was Bd growth up to the bacterial streak; or 3) indeterminate if the Bd did not grow at all anywhere on the plate or if the bacterial streak overtook the whole plate. If an indeterminate result was obtained the experiment was repeated until a negative or positive was obtained.

RESULTS

Bacterial Culture and Identification

Of the 36 bacterial isolates obtained from the eleven *L. archeyi* at the Coromandel site, 31 unique bacteria were identified from ten of the frogs. *Pseudomonas* spp. were the most common bacterial genera identified and comprised 21 of the 31 bacterial isolates (68%) (Table 1).

Of the 62 bacterial isolates obtained from the 33 *L. archeyi* at the Whareorino site, 34 unique bacteria were identified from 24 of the frogs. *Pseudomonas* spp. were again the most common genera identified and comprised 24 of the 34 isolates (71%) (Table 1).

Of the 50 bacterial isolates obtained from the twenty *L. hochstetteri* at the same Whareorino site, 31 unique bacteria were identified from 16 of the frogs. *Flavobacterium* spp. were most common genera identified and comprised 12 of the 31 bacterial isolates (39%) (Table 1).

Three isolates of *Pseudomonas* were found in more than one location (*Pseudomonas putida isolate PSB31*, *Pseudomonas sp. BR6-10* and *Pseudomonas sp. 29H)* which made the total unique isolates identified actually 92 (Table 1).

Flavobacterium species were significantly more prevalent in the Whareorino *L. hochstetteri* frogs when compared to the Whareorino *L. archeyi* (Fisher's Exact Test; P=0.02; (odds ratio 6.3 with 95% CI 1.3-33.1)) and when compared to all *L. archeyi* at both the Whareorino and Coromandel locations together (Fisher's Exact Test; P=0.01; (odds ratio 6.4 with 95% CI 1.5-29.2)).

In vitro Bd-bacterial challenge assay

The Bd-bacterial challenge assay was only performed for bacterial species from *L. archeyi* at the Coromandel location as it was difficult to obtain consistent results using the technique developed by Harris et al (2006). From 31 bacterial challenges, just one was positive, (*Flavobacterium sp. XAS590*; Figure 1); 20 were negative and ten were indeterminate despite repeated attempts to get a definitive result. The reasons for a test to be indeterminate were: 1) The Bd agar plate too dry thus killing the zoospores or; 2) The plate was not dried for long enough so some mucoid bacteria that typically tend to expand easily on a plate (e.g. *Pseudomonas*), took over the entire plate within 24 hours so that a 24 hour reading could not be obtained (Figure 2).

DISCUSSION

We isolated and identified 92 unique bacterial isolates from 64 *L. archeyi* and *L. hochstetteri* frogs in the Coromandel and Whareorino regions in New Zealand. One of these isolates, *Flavobacterium sp. XAS590*, inhibited the growth of Bd in vitro. In addition we found that *Flavobacterium* spp. occur more frequently in *L. hochstetteri* when compared to *L. archeyi*.

Baseline data on the cutaneous bacteria in healthy free- ranging *L. archeyi* and *L. hochstetteri* is valuable information that could be used to interpret bacterial culture results as part of a diagnostic work-up in sick frogs. It may also be useful when interpreting bacterial skin cultures from pre-translocation or quarantine disease screening, where abnormal results can jeopardize an entire movement of frogs. When comparing these results to those of Potter and Norman (2006), only *Serratia* spp. were found in both studies. This difference could be due to more precise molecular DNA identification techniques used in this study, (Ludwig, 2008), or reflect differences between the

bacterial flora on the dorsal and ventral skin surfaces (Culp et al., 2007). For bacterial culture we used TGhL agar plates and lower incubation temperatures to simulate conditions in wild frogs, and also those favourable to Bd growth (Berger et al., 2004), thus our methods could have selected for different bacteria than the previous study since dissimilar methods were used. We also did not identify all the bacterial isolates we cultured as the cost was too great. By grouping together morphologically similar isolates we expected to identify most of the flora. However, as bacteria are difficult to distinguish solely by gross morphology, we may have missed some species. In addition, a significant proportion of bacteria are unculturable. *Flavobacterium* XAS590 from *L. archeyi* was the only bacterial isolate that showed anti-Bd properties in our experiments. This is the first time that bacteria from *Leiopelma* spp. have been shown to exhibit *in vitro* anti-Bd properties and may be significant in explaining the apparent immunity to chytridiomycosis in these frogs. *Flavobacterium* species were also isolated significantly more in *L. hochstetteri* than *L. archeyi* in both the Whareorino location and when combining both the Coromandel and Whareorino locations. If *Flavobacterium* play a role in innate immunity against chytridiomycosis in these *L. archeyi* we would expect a higher prevalence as previous studies have shown that if a high proportion of susceptible frogs have at least one anti-Bd bacterial species present, the population can persist despite the presence of Bd (Lam, 2010; Woodhams et al., 2007). Therefore, our results indicate that *L. archeyi* from the Coromandel may not use bacterial inhibition as a principle means of defence against Bd, unless other unidentified species are inhibitory. We recommend that bacteria are tested further using the new broth challenge assay technique developed by Bell et al. (in review). This technique avoids some of the issues of the agar plate method and may provide more reliable results. *Flavobacterium* should be investigated further for its role in host resistance to Bd and added to the growing list of bacteria that can be used in potential bioaugmentation trials.

Bacterial isolates from the genus *Pseudomonas* were the most common isolate found in *L. archeyi* in both locations. Although these mucoid bacteria did not work well in our Bd-bacterial challenge, they have been successfully challenged in other studies and some species were found to have anti-Bd properties (Lam, 2010; Lauer et al., 2007; Lauer et al., 2008; Woodhams et al., 2007).

We suggest that the *Pseudomonas* isolates from New Zealand should be investigated further for Bd inhibition.

Acknowledgements: Funding for this project was provided by the Auckland Zoo Charitable Trust Conservation Fund. Many thanks to the staff at Landcare Auckland who were instrumental in the bacterial culturing, identification and storage of chytrid samples: Stanley Bellgard, Maureen Fletcher, Karen Hoksbergen, Daniel Than, Bevan Weir and Paula Wilke. Thanks also to Lisa Daglish and Amanda Haigh from the Department of Conservation who collected the bacterial samples. Many thanks to Reid Harris, Brianna Lam and Jennifer Walke for technical advice. Thanks also to the New Zealand Maori iwi for supporting native frog research.

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Table 1: Closest taxonomic affiliation from GenBank for all unique 16s rDNA sequences. Numbers of frogs possessing each unique sequence are shown by species: *Leiopelma archeyi* (La) and *Leiopelma hochstetteri* (Lh), and site: Coromandel Pahi Moehou (Coro) and Whareorino (Whare). n = the number of frogs that had bacterial isolates cultured. Some frogs had more than one bacterial isolate cultured. The * denotes the positive isolate in the Bd-bacterial challenge and the ^ denotes the negative control.

			Coro	Whare	
Taxonomy/GenBank closest match	**Accession Number**	**Similarity %**	**La n= 10**	**La n= 24**	**Lh n=28**
Bacteroidetes					
Flavobacteria					
*Chryseobacterium sp. 3Ablue^*	EU057843	98.9	1	-	-
Chryseobacterium jejunense	AB682422	99.5	1	-	-
Flavobacterium columnare	AY747592	99.4	-	1	-
Flavobacterium sp. DB 2.3-10	AM493386	99.2	-	-	1
Flavobacterium sp. EP 372	AF493653	99.8	-	-	1
Flavobacterium sp. KOPRI 25403	GU062496	92.2	-	1	-
Flavobacterium sp. KOPRI 25403	GU062496	99.1	-	1	-
Flavobacterium sp. LM-20-Fp	HE573273	97.9	-	-	1
Flavobacterium sp. Sa CS2	JQ806423	99.7	-	-	1
Flavobacterium sp. WB 3.1-53	AM934654	99.6	-	-	1
Flavobacterium sp. WB 3.1-78	AM177614	98.9	-	-	1
Flavobacterium sp. WB 3.1-79	AM934656	99.0	-	-	1
Flavobacterium sp. WB 3.2-28	AM934659	99.8	-	-	1
Flavobacterium sp. WB 3.3.45	AM177620	88.8	-	1	-
Flavobacterium sp. WB 3.4.10	AM177622	98.5	-	-	1
Flavobacterium sp. WB 4.4-22	AM177636	99.7			1
Flavobacterium sp. WB 4.3-36	AM934669	99.3			1
*Flavobacterium sp. XAS590**	GQ395239	98.5	1	-	-
Flavobacterium sp. YO51	DQ778315	99.9	1	-	-
Flavobacterium sp. III-082-7	FJ786051	99.8	-	1	
Flavobacterium sp. III-082-7	FJ786051	99.3	-	-	1
Proteobacteria					
Betaproteobacteria					
Duganella zoogloeoides strain IAM 12670	NR025833	99.5	-	1	-
Gammaproteobacteria					
Acinetobacter sp. AW 1-18	JQ316540	99.1	-	-	1
Acinetobacter sp. LB BR 12338	JQ247320	99.7	-	-	1
Acinetobacter sp. LD BR 12340	JQ247322	99.6	-	-	1
Aeromonas tecta strn. CECT7082	HQ832416	99.9	-	-	1
Aeromonas veronii strn. MTTSA 14	JQ795738	100.0	-	-	1
Aeromonas sp. F518	AJ458402	100.0	-	-	1
Enterobacteriaceae bacterium JL6J	JX162035	99.7	-	-	1
Hafnia sp. NP33	EU196322	98.9	1	-	-
Pseudomonas brenneri	AM933513	100.0	1	-	-
Pseudomonas fluorescens	AB680968	100.0	-	1	-
Pseudomonas fluorescens strn. DLJ1	FJ407181	100.0	1	-	-
Pseudomonas fluorescens strn.F32	HQ647251	100.0	-	1	-
Pseudomonas fluorescens Pf0-1	CP000094	99.9	-	1	-
Pseudomonas fragi	AB685586	99.9	1	-	-
Pseudomonas koreensis	AB495131	99.8	-	1	-
Pseudomonas palleroniana strn. POT2	JQ281539	99.9	1	-	-
Pseudomonas poae strn. BCHCNZ253	GU188947	100.0	-	1	-
Pseudomonas poae strn. YUST-DW11	HM640290	99.9	-	1	-
Pseudomonas putida	AB681704	99.7	1	-	-
Pseudomonas putida	AB681704	99.9	1		
Pseudomonas putida isolate PSB31	HQ242744	99.4	-	1	1
Pseudomonas putida isolate PSB31	HQ242744	99.6	-	-	1

Pseudomonas putida strn. CICR-GV2	JX276777	89.5	-	1	-
Pseudomonas putida strn. LCB43	JN650580	99.3	-	1	-
Pseudomonas tolaasii strn. 93	JX417438	99.8	-	1	-
Pseudomonas tolaasii strn. 93	JX417438	99.9	-	1	-
Pseudomonas vancouverensis strn. A-18	HQ202824	100.0	1	-	-
Pseudomonas sp. A8(2011)	JN228275	99.7	1	-	-
Pseudomonas sp. A8(2011)	JN228275	99.7	-	1	-
Pseudomonas sp. A8(2011)	JN228275	99.6	-	1	-
Pseudomonas sp. A8(2011)	JN228275	99.5	-	-	1
Pseudomonas sp. A17(2011)	JN228277	100.0	1	-	-
Pseudomonas sp. A17(2011)	JN228277	99.9	1	-	-
Pseudomonas sp. BCRC 80328	JQ361087	100.0	1	-	-
Pseudomonas sp. BR6-10	EU853194	100.0	1	2	-
Pseudomonas sp. CBZ-4	JQ782892	99.9	-	-	1
Pseudomonas sp. Ch313	AB289615	99.9	1	-	-
Pseudomonas sp. Ch313	AB289615	99.8	-	1	-
Pseudomonas sp. Cmc27	JQ917993	99.5	-	1	-
Pseudomonas sp. DPs-27	JQ074038	99.8	-	1	-
Pseudomonas sp. G1-21-2	EU781539	98.0	-	-	1
Pseudomonas sp. G52	FN547408	98.0	-	-	1
Pseudomonas sp. JSPB2	JQ308615	100.0	-	2	-
Pseudomonas sp. KA-26	HE979862	99.5	-	-	1
Pseudomonas sp. KBOS 17	AY653222	99.9	1	-	-
Pseudomonas sp. LD002	HQ713573	99.1	1	-	-
Pseudomonas sp. PDD-32b-42	HQ256842	90.0	-	1	-
Pseudomonas sp. RPBP7	JN411670	100.0	-	-	1
Pseudomonas sp. SaCS18	JQ806427	99.9	-	1	-
Pseudomonas sp. SGb14	HQ224617	99.8	1	-	-
Pseudomonas sp. SGb149	HQ224651	99.8	1	-	-
Pseudomonas sp. SGb149	HQ224651	99.9	1	-	-
Pseudomonas sp. SY7	EU073118	99.4	1	-	-
Pseudomonas sp. SY7	EU073118	99.5	-	1	-
Pseudomonas sp. TB2-1-II	AY599711	100.0	-	1	-
Pseudomonas sp. VS-1	JF699698	99.9	-	-	1
Pseudomonas sp. VTAE174	JN886726	99.9	1	-	-
Pseudomonas sp. 6A	DQ417331	99.9	-	1	-
Pseudomonas sp. 29H	EU057890	100.0	1	1	-
Rahnella aquatilis	GU171376	98.7	1	-	-
Rahnella aquatilis strn.2B-CDF	FJ811859	99.4	-	1	-
Rahnella sp. WMR15	AM167519	99.0	1	-	-
Rahnella sp. WMR58	AM160791	99.0	1	-	-
Serratia sp. AC-CS-1B	FJ231172	99.9	1	-	-
Serratia sp. A7	DQ103507	100.0	-	1	-
Serratia sp. D1	DQ103511	100.0	-	1	-
Serratia sp. D5	EU100389	96.7	-	-	1
Serratia sp. ORC3	JQ236628	99.9	1	-	-
Serratia sp. 136-2	EU557341	100.0	-	2	-
Stenotrophomonas rhizophila strn. AB11	JQ410475	99.8	-	-	1
Stenotrophomonas rhizophila strn. Bacteria_188	JQ800450	98.7	-	-	1
		Total unique isolates	**31**	**34**	**31**

Figure 1: Positive Bd-bacterial challenge *Flavobacterium sp. XAS590*. Note the clearing zone around the streak where Bd is not growing.

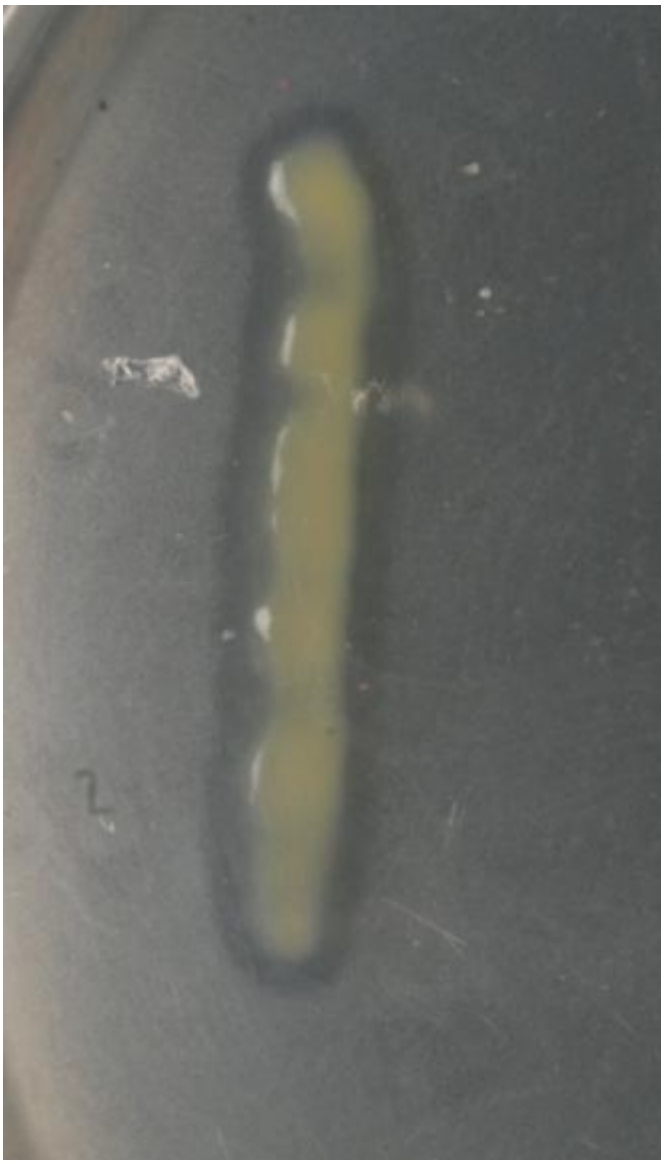
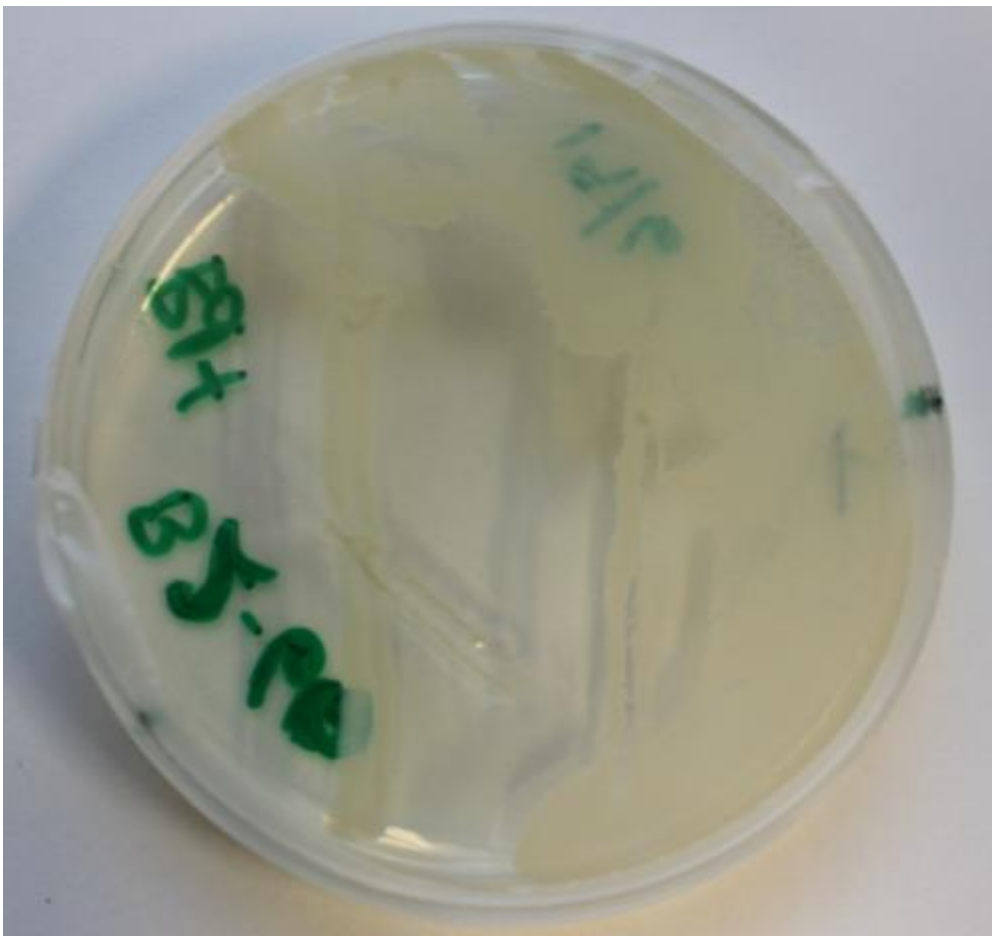


Figure 2: An example of an bacterial isolate spreading over the plate causing the result to be indeterminate.



Appendix

Appendix 1: Methods and results of the isolation and genotyping of a New Zealand isolate of *Batrachochytrium dendrobatidis*.

Bd Isolation and Genotyping

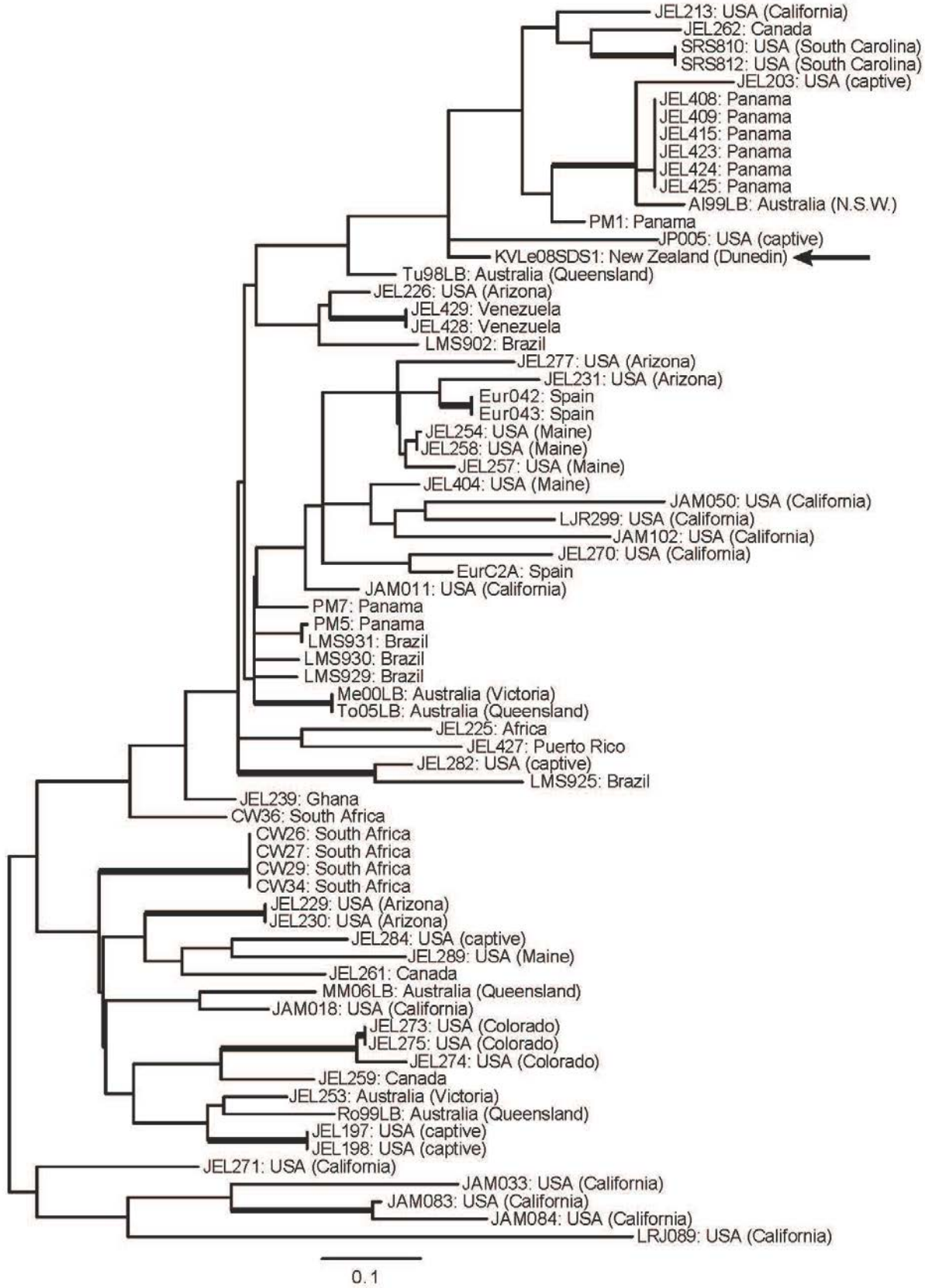
Skin samples were obtained opportunistically from the free-ranging non-native frog *Litoria ewingii* and confirmed as infected with chytridiomycosis through observation of zoosporangia or zoospores in the skin under a light microscope. Bd was cultured using established methods within a Class II biohazard cabinet (Berger et al., 2009; Longcore et al., 1999).

To characterize the NZ strain, we genotyped over 5,000 base pairs DNA using sequences from 17 nuclear SNP loci by direct PCR and sequencing as per James et al. (2009). The NZ strain data were compared to the 67 published genotypes of Schloegel et al. (2010) by generating an UPGMA dendrogram in the program PAUP* with genetic distances estimating according to the "hetequal" coding of James et al. (2009).

One Bd isolate was obtained on October 8th, 2008, from a sick wild-caught non-native frog (*Litoria ewingii)* from the Kaikourai Valley near Dunedin, New Zealand (-45.8, 170.6) and named KVLe08SDS1 per the Berger protocol (Berger et al., 2005). This isolate was cryo-archived (Boyle et al., 2003) and kept in TGhL broth per established protocols (Berger et al., 2009; Longcore et al., 1999).

A genetic tree with the NZ Bd isolate was created to compare with a global set of strains (Figure 1A). The strain had a unique multilocus genotype and lacks the diagnostic alleles at the three loci (9893X2, R6046, BdC24) that are only found in temperate North America and Europe (James et al. 2009). The closest strains to the NZ isolate are a cluster of Panamanian strains, an Australian strain from New South Wales (Alstonville-Lcaerulea-99-LB-1), and a strain isolated from a captive *Dyscophus guineti* from the Bronx Zoo, NY, USA (JEL203). The arrow points to the New Zealand isolate of *Batrachochytrium dendrobatidis*.

Figure 1A: Genetic tree with the NZ Bd isolate compared with a global set of strains



Chapter 11: Conclusions and Recommendations

This PhD project was initiated because all four native New Zealand frog species are endangered to some degree and their diseases had been largely unstudied. Reasons for past declines include habitat loss, introduced predators and a population crash in *L. archeyi* which was linked to chytridiomycosis (Bell, 2004). Attempts to establish captive breeding colonies had been unsuccessful.

The project was divided into two main questions:

1) What is the health status of captive New Zealand frogs and what diseases if any are limiting their survival? and

2) Is the amphibian chytrid a threat to free-ranging native frogs in New Zealand?

To summarize the answers:

1) Captive frogs had high mortality rates due to inadequate husbandry; and

2) Chytridiomycosis does not appear to be a current threat to wild populations.

How do these answers relate to practical frog conservation? Here I provide specific recommendations for managers, biologists and veterinarians based on outcomes of my research on wild and captive frogs in conjunction with what is currently known about the ecology and biology of leiopelmatids. I also outline management actions and priorities for disease research aimed at increasing the wild populations of native frogs.

Captive Native Frogs

Summary of Outcomes

In Chapter 2, my initial review of the husbandry and mortality rates of captive frogs from 2000-2005, showed mortality was high for captive *Leiopelma archeyi* and *Leiopelma hochstetteri*, but not *Leiopelma pakeka*. A single cause for the high mortality was not identified but, the overheating of substrate in enclosures was suspected to be a contributing factor at Canterbury University and

Auckland Zoo. Chytridiomycosis was not identified as a cause of death in any captive cases. I found mortality rates continued to be high for captive *L. archeyi* and *L. hochstetteri* from 2005-2009. In Chapters 3, 4 and 6, I reviewed the health and husbandry of *L. archeyi* and *L. hochstetteri* in greater detail and determined the main cause of mortality and poor health was metabolic bone disease (MBD) caused by an inadequate diet, a lack of ultraviolet-B (UVB) light and fluoride exposure from tap water. *Leiopelma pakeka* husbandry was not further investigated here due to the low mortality noted in Chapter 2.

Chapters 8 and 9 focus on chytridiomycosis: a transmission experiment indicated that *L. archeyi* could self-cure from amphibian chytrid and surveys showed the current wild populations appeared stable despite the presence of *Batrachochytrium dendrobatidis* (Bd). The resistance of *L.archeyi* to experimental infection is consistent with recent results from other *Leiopelma* spp. (Ohmer, 2011). The implications for captive *L. archeyi* were monumental. At the start of this project, *L. archeyi* were kept indoors to eliminate contact with the non-native *Litoria* spp. that were a possible source of amphibian chytrid. As my results showed the threat of chytridiomycosis to the captive frogs was low, *L. archeyi* could be moved outside. Outside enclosures were desirable as they facilitated the management recommendations in Chapters 3 and 4: to increase the exposure of the frogs to sunlight, and increase the diversity of their diet to prevent MBD.

Recomendations for Managers

1. Husbandry conditions in general should be based on specific knowledge of the habitat of each species. Many amphibian species, such as the leiopelmatids, are distinct and unique. Although many amphibian species share common traits, leiopelmatids have many unique morphological features and it is possible they also have unique physiological requirements.
2. Use outside enclosures where possible and if not, provide artificial UVB light. The levels and length of UVB exposure should simulate seasonal conditions from the frogs' natural habitat. Monitor monthly the UVB light received at the frog level and adjust lighting as needed. Provide choice of exposure within enclosures, including shelters.

3. Analyse mineral content and pH of the water supply monthly and use filters, additives, or alternate systems to simulate wild conditions, which may differ between species. Ensure that fluoride is removed.
4. Aim to provide a captive diet that provides similar prey composition and nutrient levels to wild diets, especially the Ca:P ratio. As this data is not available for all species, the suggested captive *Leiopelma* diet (see Chapter 3) may be a useful baseline and includes invertebrate type and size. Consider on-site cultivation of desired invertebrates.
5. Provide similar substrate within enclosures to that in natural habitats for each species. Although there is no evidence that this was a critical factor in the health of leiopelmatids, the mineral content and pH of the soil can affect the ionic movement of minerals such as calcium and chloride in the ventral "drinking patch" of some frogs (K.Whittaker, pers. comm. 2009) and so could be a factor in MBD.
6. Do not isolate frogs with adenomatous hyperplasia (AH), as it is not contagious and the presence of AH does not increase the relative risk of death (Chapter 6). The numbers of captive breeding stock of *L. archeyi* are now critically low and the optimum number per tank and sex ratio to establish captive breeding is still unknown. Therefore, until proven otherwise, all frogs from the same population should be combined to maximize their breeding potential. However, the presence of new lesions should alert managers that there is an imbalance in environmental parameters.
7. Investigate the health and husbandry of captive *L. pakeka* addressing all the parameters that were found to be an issue with *L. archeyi* and *L. hochstetteri*. This should be a priority for several reasons. One, there has been X-ray evidence showing malformed femurs in *L. pakeka* (P.Bishop and S.Shaw, unpubl. data) suggesting MBD occurs in these frogs. Secondly, if these frogs are to be enlisted in new captive reproduction programmes, for example at the Wellington Zoo and/or Orana Wildlife Park in Christchurch, they could be housed in indoor group tanks due to the cold climate. Investigations comparing free-living conditions with the current captive frogs are needed to optimize captive conditions.

Starting a new colony with ideal husbandry should ensure that mortality rates do not increase in this species.

8. Success in captive husbandry should be measured including these three parameters:
    a. A decrease in mortality. In the wild, individuals in marked recapture plots have been found up to 40 years later (Bell, 1994). In a protected environment with optimal husbandry, the captive frogs should live at least as long as their free-ranging counterparts.
    b. Success in reproduction. Reproductive females likely have higher requirements for calcium metabolism. Successful reproduction should be an indication that conditions causing metabolic bone disease have improved.
    c. Rearing of froglets to the adult phase. Leiopelmatid froglets have not been raised successfully in captivity to the adult phase which is likely due to inadequate husbandry. Following my suggestions for improved diet and water (Chapter 3) is likely to improve rearing success.

Free-Living Native Frogs

Summary of Outcomes

Chapters 7 and 8 show that Bd now appears to be endemic and widespread. Both the Coromandel and Whareorino *L. archeyi* populations are Bd positive and have had a stable prevalence (2006-2010) and frog numbers are stable (B.Bell, unpubl.data; L.Daglish, unpubl.data). Hence the Whareorino population of *L. archeyi* is not expected to decline further due to chytridiomycosis.

Populations of *L. hochstetteri*, *L hamiltoni* and *L. pakeka* have been appropriately sampled and Bd has not been found. As discussed in Chapter 8, there is still uncertainty if the isolated, island populations of *L. hamiltoni* or *L. pakeka* have ever been exposed to Bd, which may have been prevented due to the strict hygiene measures that have been in place. Recent experimental data has shown that *L. hochstetteri* has high resistance to Bd and that *L. pakeka* can self-cure and do not show clinical signs of chytridiomycosis (Ohmer, 2011). This information in conjunction with my data leads

to the speculation that Bd may not be a threat to *L hochstetteri, L. hamiltoni* and *L. pakeka*. However, as the similarly resistant *L. archeyi* did have a severe population crash when Bd entered the naïve Coromandel population (Bell, 2004), cautionary hygiene measures should be upheld as uncertainty remains regarding the threat to these island populations. Predictive distribution models show most of New Zealand has suitable climate for Bd (K.Murray, unpubl.data).

Recommendations for Managers

1. The impact of Bd on the survivability of individual frogs should be determined. Long-term mark recapture data and Bd results need analysis in both the Coromandel and Whareorino as a priority to understand the dynamics of the disease in wild populations. Although they appear be co-existing with Bd, the abundance of the Coromandel population has not fully recovered since the first decline in 1996 and it is unknown if Bd is contributing to this depression. Regular surveys of population abundance are important to monitor the stability of this species.
2. Reintroductions and translocations from stable captive and wild populations are needed to increase the number of wild populations. This would reduce their risk of extinction from catastrophic events, such as the introduction of Ranavirus, or other new diseases. To decrease the risk to *L. archeyi*, *L. hamiltoni* and *L. pakeka*, further populations should be established in both suitable off-shore and on-shore locations. There are currently only three populations of *L. archeyi* (one of which is a recent translocation), one population of *L. hamiltoni* and one population of *L. pakeka*. Although *L. hochstetteri* has over twenty populations in the North Island and considered the least endangered of the *Leiopelma* spp., translocation to an off-shore island location should also be considered to establish a protected "insurance population" against stochastic events.
3. Maintaining strict field hygiene around all wild populations is critical to prevent spread of Bd and other pathogens. Disease could potentially be introduced via researchers, fishing, bushwalkers etc.

4. Continue the mortality investigations of wild frogs, to improve knowledge of baseline diseases and as surveillance for emerging diseases. Full post-mortems need to be performed on any wild frog found dead. In the past, frogs thought to have died from a known cause such as rat predation or caught in invertebrate pitfall traps have not had post-mortems.
5. If invertebrate pitfall traps are used in native frog habitat, traps needs to be checked more frequently or changed to improve the preservation of any bycatch frogs. The bycatch frogs I examined represented healthy leiopelmatids and were an invaluable resource for baseline values, especially in disease investigations. However, due to the length of time in the suboptimal pitfall trap preservatives, many of the organs were not well preserved. Due to their endangered conservation status, the specimens' value needs to be maximised.

Closing Summary

This study has already led to the improved health in captive frogs. Metabolic bone disease is the most common disease of captive frogs and so these findings have global relevance to frog conservation efforts, as in many cases the only proven intervention against chytridiomycosis is to bring frogs into captivity.

Overall, the potential for conserving New Zealand native frogs has a positive outlook. The mainland populations of *L. archeyi* and *L. hochstetteri* appear to be stable and co-existing with the presence of Bd in their environment. However, as the small populations are still vulnerable to agents of mass decline, establishing healthy, reproducing captive collections of all leiopelmatids is important. Maintaining strict field hygiene around all wild populations is critical to prevent the spread of Bd and other pathogens.

The results of my thesis have brought together years of field work and laboratory data into one collection. I have clarified the role of chytridiomycosis in the wild populations and described the disease syndromes present in the captive populations. Diseases of New Zealand native frogs require further investigation to continue to build knowledge in this previously neglected field.

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Supplementary Material 1: New Zealand database of *Batrachochytrium dendrobatidis* infection records 1930-2010

Compiled by	Data base ID	Species	Sex	Site	Region	Country	Year	Diagnostic	# individuals	# indivs positive	Collector source	Original database	Disease status	Latitude	Longitude	Dead or sick
StephanieShaw	1	Litoria raniformis	unknown	DunstanRoadAlexandra	Otago	New Zealand	2008	SYBR green qPCR	79	0	S.Herbert	S.Shaw	negative	-45.22466	169.37958	No
StephanieShaw	2	Leiopelma hochstetteri	unknown	WaitakereRanges	Auckland	New Zealand	2008	TqPCR	1	0	V.Moreno	S.Shaw	negative	-37.00203	174.55577	No
StephanieShaw	3	Leiopelma hochstetteri	unknown	WaitakereRanges	Auckland	New Zealand	2008	TqPCR	1	0	V.Moreno	S.Shaw	negative	-37.02964	174.51625	No
StephanieShaw	4	Leiopelma hochstetteri	unknown	WaitakereRanges	Auckland	New Zealand	2008	TqPCR	1	0	V.Moreno	S.Shaw	negative	-37.02966	174.51607	No
StephanieShaw	5	Leiopelma hochstetteri	unknown	WaitakereRanges	Auckland	New Zealand	2008	TqPCR	1	0	V.Moreno	S.Shaw	negative	-36.94351	174.47645	No
StephanieShaw	6	Leiopelma hochstetteri	unknown	WaitakereRanges	Auckland	New Zealand	2008	TqPCR	1	0	V.Moreno	S.Shaw	negative	-36.94269	174.47572	No
StephanieShaw	7	Leiopelma hochstetteri	unknown	WaitakereRanges	Auckland	New Zealand	2008	TqPCR	1	0	V.Moreno	S.Shaw	negative	-36.96418	174.50342	No
StephanieShaw	8	Leiopelma hochstetteri	unknown	WaitakereRanges	Auckland	New Zealand	2008	TqPCR	1	0	V.Moreno	S.Shaw	negative	-36.96419	174.50342	No
StephanieShaw	9	Leiopelma hochstetteri	unknown	WaitakereRanges	Auckland	New Zealand	2008	TqPCR	1	0	V.Moreno	S.Shaw	negative	-36.96430	174.50331	No
StephanieShaw	10	Leiopelma hochstetteri	unknown	WaitakereRanges	Auckland	New Zealand	2008	TqPCR	1	0	V.Moreno	S.Shaw	negative	-36.94750	174.56278	No
StephanieShaw	11	Leiopelma hochstetteri	unknown	WaitakereRanges	Auckland	New Zealand	2008	TqPCR	1	0	V.Moreno	S.Shaw	negative	-36.94746	174.56310	No
StephanieShaw	12	Leiopelma hochstetteri	unknown	WaitakereRanges	Auckland	New Zealand	2008	TqPCR	1	0	V.Moreno	S.Shaw	negative	-36.94486	174.56088	No
StephanieShaw	13	Leiopelma hochstetteri	unknown	WaitakereRanges	Auckland	New Zealand	2008	TqPCR	1	0	V.Moreno	S.Shaw	negative	-36.94479	174.56085	No
StephanieShaw	14	Leiopelma hochstetteri	unknown	WaitakereRanges	Auckland	New Zealand	2008	TqPCR	1	0	V.Moreno	S.Shaw	negative	-37.00209	174.55572	No
StephanieShaw	15	Leiopelma hochstetteri	unknown	WaitakereRanges	Auckland	New Zealand	2008	TqPCR	1	0	V.Moreno	S.Shaw	negative	-37.00033	174.54563	No
StephanieShaw	16	Leiopelma hochstetteri	unknown	WaitakereRanges	Auckland	New Zealand	2008	TqPCR	1	0	V.Moreno	S.Shaw	negative	-37.00015	174.54557	No
StephanieShaw	17	Leiopelma hochstetteri	unknown	WaitakereRanges	Auckland	New Zealand	2008	TqPCR	1	0	V.Moreno	S.Shaw	negative	-37.00004	174.54564	No
StephanieShaw	18	Leiopelma hochstetteri	unknown	WaitakereRanges	Auckland	New Zealand	2008	TqPCR	1	0	V.Moreno	S.Shaw	negative	-37.02101	174.53786	No
StephanieShaw	19	Leiopelma hochstetteri	unknown	WaitakereRanges	Auckland	New Zealand	2008	TqPCR	1	0	V.Moreno	S.Shaw	negative	-37.02113	174.53798	No
StephanieShaw	20	Leiopelma hochstetteri	unknown	WaitakereRanges	Auckland	New Zealand	2008	TqPCR	1	0	V.Moreno	S.Shaw	negative	-37.02077	174.53803	No
StephanieShaw	21	Leiopelma hochstetteri	unknown	WaitakereRanges	Auckland	New Zealand	2008	TqPCR	1	0	V.Moreno	S.Shaw	negative	-37.00906	174.53079	No

StephanieShaw	22	Leiopelma hochstetteri	unknown	WaitakereRanges	Auckland	New Zealand	2008	TqPCR	1	0	V.Moreno	S.Shaw	negative	-37.00906	174.53079	No
StephanieShaw	23	Leiopelma hochstetteri	unknown	WaitakereRanges	Auckland	New Zealand	2008	TqPCR	1	0	V.Moreno	S.Shaw	negative	-37.00904	174.53077	No
StephanieShaw	24	Leiopelma hochstetteri	unknown	WaitakereRanges	Auckland	New Zealand	2008	TqPCR	1	0	V.Moreno	S.Shaw	negative	-36.94818	174.50510	No
StephanieShaw	25	Leiopelma hochstetteri	unknown	WaitakereRanges	Auckland	New Zealand	2008	TqPCR	1	0	V.Moreno	S.Shaw	negative	-36.94818	174.50512	No
StephanieShaw	26	Leiopelma hochstetteri	unknown	WaitakereRanges	Auckland	New Zealand	2008	TqPCR	1	0	V.Moreno	S.Shaw	negative	-37.00710	174.49572	No
StephanieShaw	27	Leiopelma hochstetteri	unknown	WaitakereRanges	Auckland	New Zealand	2008	TqPCR	1	0	V.Moreno	S.Shaw	negative	-37.00719	174.49573	No
StephanieShaw	28	Leiopelma hochstetteri	unknown	WaitakereRanges	Auckland	New Zealand	2008	TqPCR	1	0	V.Moreno	S.Shaw	negative	-37.00710	174.49574	No
StephanieShaw	29	Leiopelma hochstetteri	unknown	WaitakereRanges	Auckland	New Zealand	2008	TqPCR	1	0	V.Moreno	S.Shaw	negative	-36.95012	174.53027	No
StephanieShaw	30	Leiopelma hochstetteri	unknown	WaitakereRanges	Auckland	New Zealand	2008	TqPCR	1	0	V.Moreno	S.Shaw	negative	-36.95013	174.53026	No
StephanieShaw	31	Leiopelma hochstetteri	unknown	WaitakereRanges	Auckland	New Zealand	2008	TqPCR	1	0	V.Moreno	S.Shaw	negative	-36.95028	174.53018	No
StephanieShaw	32	Leiopelma hochstetteri	unknown	WaitakereRanges	Auckland	New Zealand	2008	TqPCR	1	0	V.Moreno	S.Shaw	negative	-36.95030	174.53021	No
StephanieShaw	33	Leiopelma hochstetteri	unknown	WaitakereRanges	Auckland	New Zealand	2008	TqPCR	1	0	V.Moreno	S.Shaw	negative	-36.95207	174.60886	No
StephanieShaw	34	Leiopelma hochstetteri	unknown	WaitakereRanges	Auckland	New Zealand	2008	TqPCR	1	0	V.Moreno	S.Shaw	negative	-36.99852	174.51992	No
StephanieShaw	35	Leiopelma hochstetteri	unknown	WaitakereRanges	Auckland	New Zealand	2008	TqPCR	1	0	V.Moreno	S.Shaw	negative	-37.01262	174.54829	No
StephanieShaw	36	Leiopelma hochstetteri	unknown	WaitakereRanges	Auckland	New Zealand	2008	TqPCR	1	0	V.Moreno	S.Shaw	negative	-36.99757	174.51769	No
StephanieShaw	37	Leiopelma hochstetteri	unknown	WaitakereRanges	Auckland	New Zealand	2008	TqPCR	1	0	V.Moreno	S.Shaw	negative	-36.99916	174.52049	No
StephanieShaw	38	Leiopelma hochstetteri	unknown	WaitakereRanges	Auckland	New Zealand	2008	TqPCR	1	0	V.Moreno	S.Shaw	negative	-36.99922	174.52053	No
StephanieShaw	39	Leiopelma hochstetteri	unknown	WaitakereRanges	Auckland	New Zealand	2008	TqPCR	1	0	V.Moreno	S.Shaw	negative	-36.99674	174.51878	No
StephanieShaw	40	Leiopelma hochstetteri	unknown	WaitakereRanges	Auckland	New Zealand	2008	TqPCR	1	0	V.Moreno	S.Shaw	negative	-36.99631	174.52033	No
StephanieShaw	41	Leiopelma hochstetteri	unknown	WaitakereRanges	Auckland	New Zealand	2008	TqPCR	1	0	V.Moreno	S.Shaw	negative	-36.96963	174.56966	No
StephanieShaw	42	Leiopelma hochstetteri	unknown	WaitakereRanges	Auckland	New Zealand	2008	TqPCR	1	0	V.Moreno	S.Shaw	negative	-36.96831	174.55089	No
StephanieShaw	43	Leiopelma hochstetteri	unknown	WaitakereRanges	Auckland	New Zealand	2008	TqPCR	1	0	V.Moreno	S.Shaw	negative	-36.95093	174.61699	No
StephanieShaw	44	Leiopelma hochstetteri	unknown	WaitakereRanges	Auckland	New Zealand	2008	TqPCR	1	0	V.Moreno	S.Shaw	negative	-36.95112	174.61695	No
StephanieShaw	45	Leiopelma hochstetteri	unknown	WaitakereRanges	Auckland	New Zealand	2008	TqPCR	1	0	V.Moreno	S.Shaw	negative	-37.01222	174.54822	No
StephanieShaw	46	Leiopelma hochstetteri	unknown	WaitakereRanges	Auckland	New Zealand	2008	TqPCR	1	0	V.Moreno	S.Shaw	negative	-37.01221	174.54815	No
StephanieShaw	47	Leiopelma hochstetteri	unknown	WaitakereRanges	Auckland	New Zealand	2008	TqPCR	1	0	V.Moreno	S.Shaw	negative	-36.89764	174.55913	No

StephanieShaw	48	Leiopelma hochstetteri	unknown	WaitakereRanges	Auckland	New Zealand	2008	TqPCR	1	0	V.Moreno	S.Shaw	negative	-36.89778	174.55966	No
StephanieShaw	49	Leiopelma hochstetteri	unknown	WaitakereRanges	Auckland	New Zealand	2008	TqPCR	1	0	V.Moreno	S.Shaw	negative	-36.97037	174.50504	No
StephanieShaw	50	Leiopelma hochstetteri	unknown	WaitakereRanges	Auckland	New Zealand	2008	TqPCR	1	0	V.Moreno	S.Shaw	negative	-36.97013	174.50505	No
StephanieShaw	51	Leiopelma hochstetteri	unknown	WaitakereRanges	Auckland	New Zealand	2008	TqPCR	1	0	V.Moreno	S.Shaw	negative	-36.97010	174.50491	No
StephanieShaw	52	Leiopelma hochstetteri	unknown	WaitakereRanges	Auckland	New Zealand	2008	TqPCR	1	0	V.Moreno	S.Shaw	negative	-36.97011	174.50488	No
StephanieShaw	53	Leiopelma hochstetteri	unknown	WaitakereRanges	Auckland	New Zealand	2008	TqPCR	1	0	V.Moreno	S.Shaw	negative	-36.97673	174.47900	No
StephanieShaw	54	Leiopelma hochstetteri	unknown	WaitakereRanges	Auckland	New Zealand	2008	TqPCR	1	0	V.Moreno	S.Shaw	negative	-36.96742	174.56060	No
StephanieShaw	55	Leiopelma hochstetteri	unknown	WaitakereRanges	Auckland	New Zealand	2008	TqPCR	1	0	V.Moreno	S.Shaw	negative	-36.96737	174.56061	No
StephanieShaw	56	Leiopelma hochstetteri	unknown	WaitakereRanges	Auckland	New Zealand	2008	TqPCR	1	0	V.Moreno	S.Shaw	negative	-36.98370	174.49835	No
StephanieShaw	57	Leiopelma hochstetteri	unknown	WaitakereRanges	Auckland	New Zealand	2008	TqPCR	1	0	V.Moreno	S.Shaw	negative	-36.91364	174.55830	No
StephanieShaw	58	Leiopelma hochstetteri	unknown	WaitakereRanges	Auckland	New Zealand	2008	TqPCR	1	0	V.Moreno	S.Shaw	negative	-36.91372	174.55841	No
StephanieShaw	59	Leiopelma hochstetteri	unknown	WaitakereRanges	Auckland	New Zealand	2008	TqPCR	1	0	V.Moreno	S.Shaw	negative	-36.91364	174.55833	No
StephanieShaw	60	Leiopelma hochstetteri	unknown	WaitakereRanges	Auckland	New Zealand	2008	TqPCR	1	0	V.Moreno	S.Shaw	negative	-36.91814	174.50149	No
StephanieShaw	61	Leiopelma hochstetteri	unknown	WaitakereRanges	Auckland	New Zealand	2008	TqPCR	1	0	V.Moreno	S.Shaw	negative	-36.91828	174.50144	No
StephanieShaw	62	Leiopelma hochstetteri	unknown	WaitakereRanges	Auckland	New Zealand	2008	TqPCR	1	0	V.Moreno	S.Shaw	negative	-36.93259	174.51386	No
StephanieShaw	63	Leiopelma hochstetteri	unknown	GreatBarrierIsland	Auckland	New Zealand	2008	TqPCR	1	0	V.Moreno	S.Shaw	negative	-36.07520	175.35499	No
StephanieShaw	64	Leiopelma hochstetteri	unknown	GreatBarrierIsland	Auckland	New Zealand	2008	TqPCR	1	0	V.Moreno	S.Shaw	negative	-36.07520	175.35499	No
StephanieShaw	65	Leiopelma hochstetteri	unknown	GreatBarrierIsland	Auckland	New Zealand	2008	TqPCR	1	0	V.Moreno	S.Shaw	negative	-36.07485	175.36359	No
StephanieShaw	66	Leiopelma hochstetteri	unknown	GreatBarrierIsland	Auckland	New Zealand	2008	TqPCR	1	0	V.Moreno	S.Shaw	negative	-36.07948	175.35528	No
StephanieShaw	67	Leiopelma hochstetteri	unknown	GreatBarrierIsland	Auckland	New Zealand	2008	TqPCR	1	0	V.Moreno	S.Shaw	negative	-36.07959	175.35531	No
StephanieShaw	68	Leiopelma hochstetteri	unknown	GreatBarrierIsland	Auckland	New Zealand	2008	TqPCR	1	0	V.Moreno	S.Shaw	negative	-36.07840	175.36563	No
StephanieShaw	69	Leiopelma hochstetteri	unknown	GreatBarrierIsland	Auckland	New Zealand	2008	TqPCR	1	0	V.Moreno	S.Shaw	negative	-36.07926	175.36957	No
StephanieShaw	70	Leiopelma hochstetteri	unknown	GreatBarrierIsland	Auckland	New Zealand	2008	TqPCR	1	0	V.Moreno	S.Shaw	negative	-36.07903	175.36999	No
StephanieShaw	71	Leiopelma hochstetteri	unknown	GreatBarrierIsland	Auckland	New Zealand	2008	TqPCR	1	0	V.Moreno	S.Shaw	negative	-36.07925	175.37032	No
StephanieShaw	72	Leiopelma hochstetteri	unknown	GreatBarrierIsland	Auckland	New Zealand	2008	TqPCR	1	0	V.Moreno	S.Shaw	negative	-36.07920	175.37021	No
StephanieShaw	73	Leiopelma hochstetteri	unknown	GreatBarrierIsland	Auckland	New Zealand	2009	TqPCR	1	0	V.Moreno	S.Shaw	negative	-36.08841	175.37713	No

StephanieShaw	74	Leiopelma hochstetteri	unknown	GreatBarrierIsland	Auckland	New Zealand	2009	TqPCR	1	0	V.Moreno	S.Shaw	negative	-36.08863	175.37741	No
StephanieShaw	75	Leiopelma hochstetteri	unknown	GreatBarrierIsland	Auckland	New Zealand	2009	TqPCR	1	0	V.Moreno	S.Shaw	negative	-36.08900	175.37724	No
StephanieShaw	76	Leiopelma hochstetteri	unknown	GreatBarrierIsland	Auckland	New Zealand	2009	TqPCR	1	0	V.Moreno	S.Shaw	negative	-36.08889	175.37708	No
StephanieShaw	77	Leiopelma hochstetteri	unknown	GreatBarrierIsland	Auckland	New Zealand	2009	TqPCR	1	0	V.Moreno	S.Shaw	negative	-36.08890	175.37699	No
StephanieShaw	78	Leiopelma hochstetteri	unknown	GreatBarrierIsland	Auckland	New Zealand	2009	TqPCR	1	0	V.Moreno	S.Shaw	negative	-36.08876	175.37682	No
StephanieShaw	79	Leiopelma hochstetteri	unknown	GreatBarrierIsland	Auckland	New Zealand	2009	TqPCR	1	0	V.Moreno	S.Shaw	negative	-36.07487	175.35303	No
StephanieShaw	80	Leiopelma hochstetteri	unknown	GreatBarrierIsland	Auckland	New Zealand	2009	TqPCR	1	0	V.Moreno	S.Shaw	negative	-36.07507	175.35446	No
StephanieShaw	81	Leiopelma hochstetteri	unknown	GreatBarrierIsland	Auckland	New Zealand	2009	TqPCR	1	0	V.Moreno	S.Shaw	negative	-36.07508	175.35458	No
StephanieShaw	82	Leiopelma hochstetteri	unknown	GreatBarrierIsland	Auckland	New Zealand	2009	TqPCR	1	0	V.Moreno	S.Shaw	negative	-36.07487	175.35434	No
StephanieShaw	83	Leiopelma hochstetteri	unknown	GreatBarrierIsland	Auckland	New Zealand	2009	TqPCR	1	0	V.Moreno	S.Shaw	negative	-36.07502	175.35444	No
StephanieShaw	84	Leiopelma hochstetteri	unknown	GreatBarrierIsland	Auckland	New Zealand	2009	TqPCR	1	0	V.Moreno	S.Shaw	negative	-36.07434	175.35626	No
StephanieShaw	85	Leiopelma hochstetteri	unknown	GreatBarrierIsland	Auckland	New Zealand	2009	TqPCR	1	0	V.Moreno	S.Shaw	negative	-36.07424	175.35629	No
StephanieShaw	86	Leiopelma hochstetteri	unknown	GreatBarrierIsland	Auckland	New Zealand	2009	TqPCR	1	0	V.Moreno	S.Shaw	negative	-36.07413	175.35625	No
StephanieShaw	87	Leiopelma hochstetteri	unknown	GreatBarrierIsland	Auckland	New Zealand	2009	TqPCR	1	0	V.Moreno	S.Shaw	negative	-36.07503	175.36245	No
StephanieShaw	88	Leiopelma hochstetteri	unknown	GreatBarrierIsland	Auckland	New Zealand	2009	TqPCR	1	0	V.Moreno	S.Shaw	negative	-36.07427	175.36256	No
StephanieShaw	89	Leiopelma hochstetteri	unknown	GreatBarrierIsland	Auckland	New Zealand	2009	TqPCR	1	0	V.Moreno	S.Shaw	negative	-36.07482	175.36250	No
StephanieShaw	90	Leiopelma hochstetteri	unknown	GreatBarrierIsland	Auckland	New Zealand	2009	TqPCR	1	0	V.Moreno	S.Shaw	negative	-36.10037	175.39157	No
StephanieShaw	91	Leiopelma hochstetteri	unknown	GreatBarrierIsland	Auckland	New Zealand	2009	TqPCR	1	0	V.Moreno	S.Shaw	negative	-36.10136	175.39128	No
StephanieShaw	92	Leiopelma hochstetteri	unknown	GreatBarrierIsland	Auckland	New Zealand	2009	TqPCR	1	0	V.Moreno	S.Shaw	negative	-36.10139	175.39134	No
StephanieShaw	93	Leiopelma hochstetteri	unknown	GreatBarrierIsland	Auckland	New Zealand	2009	TqPCR	1	0	V.Moreno	S.Shaw	negative	-36.10160	175.39143	No
StephanieShaw	94	Leiopelma hochstetteri	unknown	GreatBarrierIsland	Auckland	New Zealand	2009	TqPCR	1	0	V.Moreno	S.Shaw	negative	-36.10176	175.39138	No
StephanieShaw	95	Leiopelma hochstetteri	unknown	GreatBarrierIsland	Auckland	New Zealand	2009	TqPCR	1	0	V.Moreno	S.Shaw	negative	-36.10194	175.39150	No
StephanieShaw	96	Leiopelma hochstetteri	unknown	GreatBarrierIsland	Auckland	New Zealand	2009	TqPCR	1	0	V.Moreno	S.Shaw	negative	-36.10174	175.39263	No
StephanieShaw	97	Leiopelma hochstetteri	unknown	GreatBarrierIsland	Auckland	New Zealand	2009	TqPCR	1	0	V.Moreno	S.Shaw	negative	-36.10180	175.39252	No
StephanieShaw	98	Leiopelma hochstetteri	unknown	GreatBarrierIsland	Auckland	New Zealand	2009	TqPCR	1	0	V.Moreno	S.Shaw	negative	-36.10175	175.39269	No
StephanieShaw	99	Leiopelma hochstetteri	unknown	GreatBarrierIsland	Auckland	New Zealand	2009	TqPCR	1	0	V.Moreno	S.Shaw	negative	-36.10152	175.39279	No

StephanieShaw	100	Leiopelma hochstetteri	unknown	GreatBarrierIsland	Auckland	New Zealand	2009	TqPCR	1	0	V.Moreno	S.Shaw	negative	-36.10152	175.39305	No
StephanieShaw	101	Leiopelma hochstetteri	unknown	GreatBarrierIsland	Auckland	New Zealand	2009	TqPCR	1	0	V.Moreno	S.Shaw	negative	-36.07358	175.37988	No
StephanieShaw	102	Leiopelma hochstetteri	unknown	GreatBarrierIsland	Auckland	New Zealand	2009	TqPCR	1	0	V.Moreno	S.Shaw	negative	-36.07351	175.37999	No
StephanieShaw	103	Leiopelma hochstetteri	unknown	GreatBarrierIsland	Auckland	New Zealand	2009	TqPCR	1	0	V.Moreno	S.Shaw	negative	-36.07351	175.37999	No
StephanieShaw	104	Leiopelma hochstetteri	unknown	GreatBarrierIsland	Auckland	New Zealand	2009	TqPCR	1	0	V.Moreno	S.Shaw	negative	-36.07363	175.38003	No
StephanieShaw	105	Leiopelma hochstetteri	unknown	GreatBarrierIsland	Auckland	New Zealand	2009	TqPCR	1	0	V.Moreno	S.Shaw	negative	-36.07386	175.38035	No
StephanieShaw	106	Leiopelma hochstetteri	unknown	GreatBarrierIsland	Auckland	New Zealand	2009	TqPCR	1	0	V.Moreno	S.Shaw	negative	-36.07385	175.38035	No
StephanieShaw	107	Leiopelma hochstetteri	unknown	GreatBarrierIsland	Auckland	New Zealand	2009	TqPCR	1	0	V.Moreno	S.Shaw	negative	-36.07356	175.37996	No
StephanieShaw	108	Leiopelma hochstetteri	unknown	GreatBarrierIsland	Auckland	New Zealand	2009	TqPCR	1	0	V.Moreno	S.Shaw	negative	-36.07365	175.38001	No
StephanieShaw	109	Leiopelma hochstetteri	unknown	GreatBarrierIsland	Auckland	New Zealand	2009	TqPCR	1	0	V.Moreno	S.Shaw	negative	-36.07375	175.38039	No
StephanieShaw	110	Leiopelma hochstetteri	unknown	GreatBarrierIsland	Auckland	New Zealand	2009	TqPCR	1	0	V.Moreno	S.Shaw	negative	-36.07362	175.38000	No
StephanieShaw	111	Leiopelma hochstetteri	unknown	GreatBarrierIsland	Auckland	New Zealand	2009	TqPCR	1	0	V.Moreno	S.Shaw	negative	-36.07362	175.38000	No
StephanieShaw	112	Leiopelma hochstetteri	unknown	GreatBarrierIsland	Auckland	New Zealand	2009	TqPCR	1	0	V.Moreno	S.Shaw	negative	-36.07360	175.38011	No
StephanieShaw	113	Leiopelma hochstetteri	unknown	GreatBarrierIsland	Auckland	New Zealand	2009	TqPCR	1	0	V.Moreno	S.Shaw	negative	-36.07380	175.38030	No
StephanieShaw	114	Leiopelma hochstetteri	unknown	GreatBarrierIsland	Auckland	New Zealand	2009	TqPCR	1	0	V.Moreno	S.Shaw	negative	-36.07374	175.38023	No
StephanieShaw	115	Leiopelma hochstetteri	unknown	GreatBarrierIsland	Auckland	New Zealand	2009	TqPCR	1	0	V.Moreno	S.Shaw	negative	-36.07373	175.38023	No
StephanieShaw	116	Leiopelma hochstetteri	unknown	GreatBarrierIsland	Auckland	New Zealand	2009	TqPCR	1	0	V.Moreno	S.Shaw	negative	-36.07361	175.38055	No
StephanieShaw	117	Leiopelma hochstetteri	unknown	GreatBarrierIsland	Auckland	New Zealand	2009	TqPCR	1	0	V.Moreno	S.Shaw	negative	-36.07377	175.38027	No
StephanieShaw	118	Leiopelma hochstetteri	unknown	GreatBarrierIsland	Auckland	New Zealand	2009	TqPCR	1	0	V.Moreno	S.Shaw	negative	-36.07371	175.38029	No
StephanieShaw	119	Leiopelma hochstetteri	unknown	GreatBarrierIsland	Auckland	New Zealand	2009	TqPCR	1	0	V.Moreno	S.Shaw	negative	-36.07377	175.38027	No
StephanieShaw	120	Leiopelma hochstetteri	unknown	GreatBarrierIsland	Auckland	New Zealand	2009	TqPCR	1	0	V.Moreno	S.Shaw	negative	-36.07372	175.38019	No
StephanieShaw	121	Leiopelma hochstetteri	unknown	GreatBarrierIsland	Auckland	New Zealand	2009	TqPCR	1	0	V.Moreno	S.Shaw	negative	-36.18378	175.39819	No
StephanieShaw	122	Leiopelma hochstetteri	unknown	GreatBarrierIsland	Auckland	New Zealand	2009	TqPCR	1	0	V.Moreno	S.Shaw	negative	-36.18378	175.39829	No
StephanieShaw	123	Leiopelma hochstetteri	unknown	GreatBarrierIsland	Auckland	New Zealand	2009	TqPCR	1	0	V.Moreno	S.Shaw	negative	-36.18382	175.39833	No
StephanieShaw	124	Leiopelma hochstetteri	unknown	GreatBarrierIsland	Auckland	New Zealand	2009	TqPCR	1	0	V.Moreno	S.Shaw	negative	-36.18391	175.39827	No
StephanieShaw	125	Leiopelma hochstetteri	unknown	GreatBarrierIsland	Auckland	New Zealand	2009	TqPCR	1	0	V.Moreno	S.Shaw	negative	-36.18397	175.39832	No

StephanieShaw	126	Leiopelma hochstetteri	unknown	HunuaRanges	Auckland	New Zealand	2006	TqPCR	3	0	S.Shaw	S.Shaw	negative	-37.1083	175.228554	No
StephanieShaw	127	Leiopelma hochstetteri	unknown	HunuaRanges	Auckland	New Zealand	2006	TqPCR	2	0	S.Shaw	S.Shaw	negative	-37.0882	175.1731	No
StephanieShaw	128	Leiopelma hochstetteri	unknown	HunuaRanges	Auckland	New Zealand	2006	TqPCR	1	0	S.Shaw	S.Shaw	negative	-37.0901	175.1729	No
StephanieShaw	129	Leiopelma hochstetteri	unknown	HunuaRanges	Auckland	New Zealand	2006	TqPCR	3	0	S.Shaw	S.Shaw	negative	-37.0904	175.1653	No
StephanieShaw	130	Leiopelma hochstetteri	unknown	HunuaRanges	Auckland	New Zealand	2006	TqPCR	2	0	S.Shaw	S.Shaw	negative	-37.0109	175.2229	No
StephanieShaw	131	Leiopelma hochstetteri	unknown	HunuaRanges	Auckland	New Zealand	2006	TqPCR	4	0	S.Shaw	S.Shaw	negative	-37.0174	175.2273	No
StephanieShaw	132	Leiopelma hochstetteri	unknown	HunuaRanges	Auckland	New Zealand	2006	TqPCR	2	0	S.Shaw	S.Shaw	negative	-37.1018	175.1860	No
StephanieShaw	133	Leiopelma hochstetteri	unknown	HunuaRanges	Auckland	New Zealand	2006	TqPCR	1	0	S.Shaw	S.Shaw	negative	-37.1068	175.1868	No
StephanieShaw	134	Leiopelma hochstetteri	unknown	HunuaRanges	Auckland	New Zealand	2006	TqPCR	2	0	S.Shaw	S.Shaw	negative	-37.1039	175.1877	No
StephanieShaw	135	Leiopelma hochstetteri	unknown	HunuaRanges	Auckland	New Zealand	2006	TqPCR	3	0	S.Shaw	S.Shaw	negative	-37.0573	175.2046	No
StephanieShaw	136	Leiopelma hochstetteri	unknown	HunuaRanges	Auckland	New Zealand	2006	TqPCR	2	0	S.Shaw	S.Shaw	negative	-37.0542	175.2039	No
StephanieShaw	137	Leiopelma hochstetteri	unknown	HunuaRanges	Auckland	New Zealand	2006	TqPCR	2	0	S.Shaw	S.Shaw	negative	-37.0524	175.2110	No
StephanieShaw	138	Leiopelma hochstetteri	unknown	HunuaRanges	Auckland	New Zealand	2006	TqPCR	1	0	S.Shaw	S.Shaw	negative	-37.0827	175.0979	No
StephanieShaw	139	Leiopelma hochstetteri	unknown	HunuaRanges	Auckland	New Zealand	2006	TqPCR	3	0	S.Shaw	S.Shaw	negative	-37.0565	175.2121	No
StephanieShaw	140	Leiopelma hochstetteri	unknown	HunuaRanges	Auckland	New Zealand	2006	TqPCR	1	0	S.Shaw	S.Shaw	negative	-37.0707	175.2181	No
StephanieShaw	141	Leiopelma hochstetteri	unknown	HunuaRanges	Auckland	New Zealand	2006	TqPCR	3	0	S.Shaw	S.Shaw	negative	-37.0857	175.2186	No
StephanieShaw	142	Leiopelma hochstetteri	unknown	HunuaRanges	Auckland	New Zealand	2006	TqPCR	1	0	S.Shaw	S.Shaw	negative	-37.0691	175.2266	No
StephanieShaw	143	Leiopelma archeyi	unknown	Whareorino	Waikato	New Zealand	2006	TqPCR	1	0	S.Shaw	S.Shaw	negative	-38.4063	174.7979	No
StephanieShaw	144	Leiopelma archeyi	unknown	Whareorino	Waikato	New Zealand	2006	TqPCR	3	0	S.Shaw	S.Shaw	negative	-38.4063	174.7981	No
StephanieShaw	145	Leiopelma archeyi	unknown	Whareorino	Waikato	New Zealand	2006	TqPCR	1	0	S.Shaw	S.Shaw	negative	-38.4078	174.8003	No
StephanieShaw	146	Leiopelma archeyi	unknown	Whareorino	Waikato	New Zealand	2006	TqPCR	4	0	S.Shaw	S.Shaw	negative	-38.4072	174.8020	No
StephanieShaw	147	Leiopelma hochstetteri	unknown	Whareorino	Waikato	New Zealand	2006	TqPCR	4	0	S.Shaw	S.Shaw	negative	-38.4035	174.7958	No
StephanieShaw	148	Leiopelma archeyi	unknown	Whareorino	Waikato	New Zealand	2006	TqPCR	3	0	S.Shaw	S.Shaw	negative	-38.4076	174.8015	No
StephanieShaw	149	Leiopelma archeyi	unknown	Whareorino	Waikato	New Zealand	2006	TqPCR	5	0	S.Shaw	S.Shaw	negative	-38.4067	174.7903	No
StephanieShaw	150	Leiopelma archeyi	unknown	Whareorino	Waikato	New Zealand	2006	TqPCR	4	0	S.Shaw	S.Shaw	negative	-38.4067	174.7903	No
StephanieShaw	151	Leiopelma archeyi	unknown	Whareorino	Waikato	New Zealand	2006	TqPCR	3	0	S.Shaw	S.Shaw	negative	-38.4059	174.7912	No

StephanieShaw	152	Leiopelma archeyi	unknown	Whareorino	Waikato	New Zealand	2006	TqPCR	3	0	S.Shaw	S.Shaw	negative	-38.4059	174.7912	No
StephanieShaw	153	Leiopelma archeyi	unknown	Whareorino	Waikato	New Zealand	2006	TqPCR	1	0	S.Shaw	S.Shaw	negative	-38.4061	174.7918	No
StephanieShaw	154	Leiopelma archeyi	unknown	Whareorino	Waikato	New Zealand	2006	TqPCR	1	0	S.Shaw	S.Shaw	negative	-38.4056	174.7922	No
StephanieShaw	155	Leiopelma archeyi	unknown	Whareorino	Waikato	New Zealand	2006	TqPCR	4	0	S.Shaw	S.Shaw	negative	-38.4074	174.8000	No
StephanieShaw	156	Leiopelma archeyi	unknown	Whareorino	Waikato	New Zealand	2006	TqPCR	2	0	S.Shaw	S.Shaw	negative	-38.4078	174.8013	No
StephanieShaw	157	Leiopelma archeyi	unknown	Whareorino	Waikato	New Zealand	2006	TqPCR	4	0	S.Shaw	S.Shaw	negative	-38.4076	174.8017	No
StephanieShaw	158	Leiopelma archeyi	unknown	Whareorino	Waikato	New Zealand	2006	TqPCR	1	0	S.Shaw	S.Shaw	negative	-38.4018	174.8019	No
StephanieShaw	159	Leiopelma archeyi	unknown	Whareorino	Waikato	New Zealand	2006	TqPCR	4	0	S.Shaw	S.Shaw	negative	-38.3880	174.7864	No
StephanieShaw	160	Leiopelma archeyi	unknown	Whareorino	Waikato	New Zealand	2006	TqPCR	3	0	S.Shaw	S.Shaw	negative	-38.3877	174.7864	No
StephanieShaw	161	Leiopelma archeyi	unknown	Whareorino	Waikato	New Zealand	2006	TqPCR	3	0	S.Shaw	S.Shaw	negative	-38.3877	174.7864	No
StephanieShaw	162	Leiopelma archeyi	unknown	Whareorino	Waikato	New Zealand	2006	TqPCR	4	0	S.Shaw	S.Shaw	negative	-38.3873	174.7864	No
StephanieShaw	163	Leiopelma archeyi	unknown	Whareorino	Waikato	New Zealand	2006	TqPCR	3	0	S.Shaw	S.Shaw	negative	-38.3873	174.7864	No
StephanieShaw	164	Leiopelma archeyi	unknown	Whareorino	Waikato	New Zealand	2006	TqPCR	4	0	S.Shaw	S.Shaw	negative	-38.3882	174.7865	No
StephanieShaw	165	Leiopelma archeyi	unknown	Whareorino	Waikato	New Zealand	2006	TqPCR	4	0	S.Shaw	S.Shaw	negative	-38.3884	174.7866	No
StephanieShaw	166	Leiopelma archeyi	unknown	Whareorino	Waikato	New Zealand	2006	TqPCR	4	0	S.Shaw	S.Shaw	negative	-38.3870	174.7865	No
StephanieShaw	167	Leiopelma archeyi	unknown	Whareorino	Waikato	New Zealand	2006	TqPCR	4	4	S.Shaw	S.Shaw	positive	-38.3872	174.7874	No
StephanieShaw	168	Leiopelma archeyi	unknown	Whareorino	Waikato	New Zealand	2006	TqPCR	3	0	S.Shaw	S.Shaw	negative	-38.4022	174.7820	No
StephanieShaw	169	Leiopelma archeyi	unknown	Whareorino	Waikato	New Zealand	2006	TqPCR	2	0	S.Shaw	S.Shaw	negative	-38.4022	174.7820	No
StephanieShaw	170	Leiopelma archeyi	unknown	Whareorino	Waikato	New Zealand	2006	TqPCR	1	0	S.Shaw	S.Shaw	negative	-38.4018	174.7820	No
StephanieShaw	171	Leiopelma archeyi	unknown	Whareorino	Waikato	New Zealand	2006	TqPCR	3	0	S.Shaw	S.Shaw	negative	-38.4019	174.7823	No
StephanieShaw	172	Leiopelma archeyi	unknown	Whareorino	Waikato	New Zealand	2006	TqPCR	1	0	S.Shaw	S.Shaw	negative	-38.4016	174.7824	No
StephanieShaw	173	Leiopelma archeyi	unknown	Whareorino	Waikato	New Zealand	2006	TqPCR	2	2	S.Shaw	S.Shaw	positive	-38.3998	174.7839	No
StephanieShaw	174	Leiopelma archeyi	unknown	Whareorino	Waikato	New Zealand	2006	TqPCR	4	4	S.Shaw	S.Shaw	positive	-38.4003	174.7833	No
StephanieShaw	175	Leiopelma archeyi	unknown	Whareorino	Waikato	New Zealand	2006	TqPCR	4	0	S.Shaw	S.Shaw	negative	-38.3996	174.7969	No
StephanieShaw	176	Leiopelma archeyi	unknown	Whareorino	Waikato	New Zealand	2006	TqPCR	3	0	S.Shaw	S.Shaw	positive	-38.3993	174.7970	No
StephanieShaw	177	Leiopelma archeyi	unknown	Whareorino	Waikato	New Zealand	2006	TqPCR	3	3	S.Shaw	S.Shaw	negative	-38.3988	174.7971	No

StephanieShaw	178	Leiopelma archeyi	unknown	Whareorino	Waikato	New Zealand	2006	TqPCR	4	0	S.Shaw	S.Shaw	negative	-38.4071	174.7975	No
StephanieShaw	179	Leiopelma archeyi	unknown	Whareorino	Waikato	New Zealand	2006	TqPCR	1	0	S.Shaw	S.Shaw	negative	-38.3981	174.7922	No
StephanieShaw	180	Leiopelma archeyi	unknown	Whareorino	Waikato	New Zealand	2006	TqPCR	1	0	S.Shaw	S.Shaw	negative	-38.3981	174.7922	No
StephanieShaw	181	Leiopelma archeyi	unknown	Whareorino	Waikato	New Zealand	2006	TqPCR	1	0	S.Shaw	S.Shaw	negative	-38.3972	174.7926	No
StephanieShaw	182	Leiopelma archeyi	unknown	Whareorino	Waikato	New Zealand	2006	TqPCR	1	0	S.Shaw	S.Shaw	negative	-38.4007	174.7940	No
StephanieShaw	183	Leiopelma archeyi	unknown	Whareorino	Waikato	New Zealand	2006	TqPCR	4	0	S.Shaw	S.Shaw	negative	-38.3985	174.7972	No
StephanieShaw	184	Leiopelma archeyi	unknown	Whareorino	Waikato	New Zealand	2006	TqPCR	1	0	S.Shaw	S.Shaw	negative	-38.3983	174.7972	No
StephanieShaw	185	Leiopelma archeyi	unknown	Whareorino	Waikato	New Zealand	2006	TqPCR	4	0	S.Shaw	S.Shaw	negative	-38.3981	174.7973	No
StephanieShaw	186	Leiopelma archeyi	unknown	Whareorino	Waikato	New Zealand	2006	TqPCR	4	0	S.Shaw	S.Shaw	negative	-38.3980	174.7973	No
StephanieShaw	187	Leiopelma archeyi	unknown	Whareorino	Waikato	New Zealand	2006	TqPCR	3	0	S.Shaw	S.Shaw	negative	-38.3977	174.7973	No
StephanieShaw	188	Leiopelma archeyi	unknown	Whareorino	Waikato	New Zealand	2006	TqPCR	3	0	S.Shaw	S.Shaw	negative	-38.3976	174.7975	No
StephanieShaw	189	Leiopelma archeyi	unknown	Whareorino	Waikato	New Zealand	2006	TqPCR	2	0	S.Shaw	S.Shaw	negative	-38.3974	174.7976	No
StephanieShaw	190	Leiopelma hochstetteri	unknown	Whareorino	Waikato	New Zealand	2006	TqPCR	4	0	S.Shaw	S.Shaw	negative	-38.4071	174.7975	no
StephanieShaw	191	Leiopelma hochstetteri	unknown	Whareorino	Waikato	New Zealand	2006	TqPCR	1	0	S.Shaw	S.Shaw	negative	-38.3981	174.7922	no
StephanieShaw	192	Leiopelma hochstetteri	unknown	Whareorino	Waikato	New Zealand	2006	TqPCR	3	0	S.Shaw	S.Shaw	negative	-38.4081	174.7956	No
StephanieShaw	193	Leiopelma hochstetteri	unknown	Whareorino	Waikato	New Zealand	2006	TqPCR	3	0	S.Shaw	S.Shaw	negative	-38.4081	174.7956	No
StephanieShaw	194	Leiopelma hochstetteri	unknown	Whareorino	Waikato	New Zealand	2006	TqPCR	3	0	S.Shaw	S.Shaw	negative	-38.4081	174.7956	No
StephanieShaw	195	Leiopelma hochstetteri	unknown	Whareorino	Waikato	New Zealand	2006	TqPCR	4	0	S.Shaw	S.Shaw	negative	-38.4081	174.7956	No
StephanieShaw	196	Leiopelma hochstetteri	unknown	Whareorino	Waikato	New Zealand	2006	TqPCR	4	0	S.Shaw	S.Shaw	negative	-38.4058	174.7917	No
StephanieShaw	197	Leiopelma hochstetteri	unknown	Whareorino	Waikato	New Zealand	2006	TqPCR	4	0	S.Shaw	S.Shaw	negative	-38.4058	174.7917	No
StephanieShaw	198	Leiopelma hochstetteri	unknown	Whareorino	Waikato	New Zealand	2006	TqPCR	1	0	S.Shaw	S.Shaw	negative	-38.4055	174.7926	No
StephanieShaw	199	Leiopelma hochstetteri	unknown	Whareorino	Waikato	New Zealand	2006	TqPCR	3	0	S.Shaw	S.Shaw	negative	-38.4049	174.7962	No
StephanieShaw	200	Leiopelma hochstetteri	unknown	Whareorino	Waikato	New Zealand	2006	TqPCR	3	0	S.Shaw	S.Shaw	negative	-38.4049	174.7962	No
StephanieShaw	201	Leiopelma hochstetteri	unknown	Whareorino	Waikato	New Zealand	2006	TqPCR	3	0	S.Shaw	S.Shaw	negative	-38.4049	174.7962	No
StephanieShaw	202	Leiopelma hochstetteri	unknown	Whareorino	Waikato	New Zealand	2006	TqPCR	2	0	S.Shaw	S.Shaw	negative	-38.4040	174.7998	No
StephanieShaw	203	Leiopelma hochstetteri	unknown	Whareorino	Waikato	New Zealand	2006	TqPCR	3	0	S.Shaw	S.Shaw	negative	-38.4028	174.8018	No

StephanieShaw	204	Leiopelma hochstetteri	unknown	Whareorino	Waikato	New Zealand	2006	TqPCR	3	0	S.Shaw	S.Shaw	negative	-38.4028	174.8018	No
StephanieShaw	205	Leiopelma hochstetteri	unknown	Whareorino	Waikato	New Zealand	2006	TqPCR	1	0	S.Shaw	S.Shaw	negative	-38.4036	174.8021	No
StephanieShaw	206	Leiopelma hochstetteri	unknown	Whareorino	Waikato	New Zealand	2006	TqPCR	2	0	S.Shaw	S.Shaw	negative	-38.4053	174.7882	No
StephanieShaw	207	Leiopelma hochstetteri	unknown	Whareorino	Waikato	New Zealand	2006	TqPCR	1	0	S.Shaw	S.Shaw	negative	-38.4070	174.7940	No
StephanieShaw	208	Leiopelma archeyi	unknown	Whareorino	Waikato	New Zealand	2006	TqPCR	1	0	S.Shaw	S.Shaw	negative	-38.4016	174.7950	No
StephanieShaw	209	Leiopelma hochstetteri	unknown	Whareorino	Waikato	New Zealand	2006	TqPCR	4	0	S.Shaw	S.Shaw	negative	-38.4079	174.7975	No
StephanieShaw	210	Leiopelma hochstetteri	unknown	Whareorino	Waikato	New Zealand	2006	TqPCR	4	0	S.Shaw	S.Shaw	negative	-38.4060	174.7974	No
StephanieShaw	211	Leiopelma hochstetteri	unknown	Raukumera	Gisborne	New Zealand	2006	TqPCR	2	0	K.Delaney	S.Shaw	negative	-37.65548	178.242382	No
StephanieShaw	212	Litoria raniformis	male	Hawera WheatleyDowns	Taranaki	New Zealand	2007	SYBR green qPCR	1	0	S.Melzer	S.Shaw	negative	-39.54573	174.35534	No
StephanieShaw	213	Litoria raniformis	male	Hawera WheatleyDowns	Taranaki	New Zealand	2007	SYBR green qPCR	1	0	S.Melzer	S.Shaw	negative	-39.54573	174.35534	No
StephanieShaw	214	Litoria raniformis	female	Hawera WheatleyDowns	Taranaki	New Zealand	2007	SYBR green qPCR	1	0	S.Melzer	S.Shaw	negative	-39.54573	174.35534	No
StephanieShaw	215	Litoria raniformis	male	Hawera WheatleyDowns	Taranaki	New Zealand	2007	SYBR green qPCR	1	0	S.Melzer	S.Shaw	negative	-39.54573	174.35534	No
StephanieShaw	216	Litoria raniformis	female	NewPlymouth Brooklands	Taranaki	New Zealand	2007	SYBR green qPCR	1	0	S.Melzer	S.Shaw	negative	-39.06789	174.08336	No
StephanieShaw	217	Litoria raniformis	unknown	NewPlymouth Brooklands	Taranaki	New Zealand	2007	SYBR green qPCR	1	0	S.Melzer	S.Shaw	negative	-39.06789	174.08336	No
StephanieShaw	218	Litoria ewingii	unknown	NewPlymouth Brooklands	Taranaki	New Zealand	2007	SYBR green qPCR	1	0	S.Melzer	S.Shaw	negative	-39.06789	174.08336	No
StephanieShaw	219	Litoria raniformis	male	NewPlymouth Brooklands	Taranaki	New Zealand	2007	SYBR green qPCR	5	0	S.Melzer	S.Shaw	negative	-39.14723	173.93542	No
StephanieShaw	220	Litoria raniformis	unknown	NewPlymouth Brooklands	Taranaki	New Zealand	2007	SYBR green qPCR	1	0	S.Melzer	S.Shaw	negative	-39.14723	173.93542	No
StephanieShaw	221	Litoria ewingii	male	NewPlymouth Brooklands	Taranaki	New Zealand	2007	SYBR green qPCR	1	0	S.Melzer	S.Shaw	negative	-39.05977	174.08419	No
StephanieShaw	222	Litoria ewingii	male	NewPlymouth Brooklands	Taranaki	New Zealand	2007	SYBR green qPCR	1	0	S.Melzer	S.Shaw	negative	-39.05977	174.08419	No
StephanieShaw	223	Litoria ewingii	male	NewPlymouth Brooklands	Taranaki	New Zealand	2007	SYBR green qPCR	1	0	S.Melzer	S.Shaw	negative	-39.05977	174.08419	No
StephanieShaw	224	Litoria ewingii	male	NewPlymouth Brooklands	Taranaki	New Zealand	2007	SYBR green qPCR	1	0	S.Melzer	S.Shaw	negative	-39.05977	174.08419	No
StephanieShaw	225	Litoria ewingii	male	NewPlymouth Brooklands	Taranaki	New Zealand	2007	SYBR green qPCR	1	0	S.Melzer	S.Shaw	negative	-39.05977	174.08419	No
StephanieShaw	226	Litoria ewingii	male	NewPlymouth Brooklands	Taranaki	New Zealand	2007	SYBR green qPCR	1	0	S.Melzer	S.Shaw	negative	-39.05977	174.08419	No
StephanieShaw	227	Litoria ewingii	male	NewPlymouth CameronSt	Taranaki	New Zealand	2007	SYBR green qPCR	1	0	S.Melzer	S.Shaw	negative	-39.05977	174.08419	No

StephanieShaw	228	Litoria aurea	male	Waitomo	Waikato	New Zealand	2007	SYBR green qPCR	1	0	S.Melzer	S.Shaw	negative	-38.26253	175.09147	No
StephanieShaw	229	Litoria aurea	female	TeKuiti	Waikato	New Zealand	2007	SYBR green qPCR	1	0	S.Melzer	S.Shaw	negative	-38.30625	175.15325	No
StephanieShaw	230	Litoria aurea	male	TeKuiti	Waikato	New Zealand	2007	SYBR green qPCR	2	0	S.Melzer	S.Shaw	negative	-38.30625	175.15325	No
StephanieShaw	231	Litoria aurea	female	Waitomo	Waikato	New Zealand	2007	SYBR green qPCR	1	0	S.Melzer	S.Shaw	negative	-38.27355	174.99436	No
StephanieShaw	232	Litoria aurea	unknown	Waitomo	Waikato	New Zealand	2007	SYBR green qPCR	1	0	S.Melzer	S.Shaw	negative	-38.27355	174.99436	No
StephanieShaw	233	Litoria aurea	male	Hamilton	Waikato	New Zealand	2007	SYBR green qPCR	1	0	S.Melzer	S.Shaw	negative	-38.26420	175.02366	No
StephanieShaw	234	Litoria aurea	male	Waihi	Waikato	New Zealand	2007	SYBR green qPCR	2	0	S.Melzer	S.Shaw	negative	-37.42086	175.80252	No
StephanieShaw	235	Leiopelma archeyi	unknown	KomataReefs	Waikato	New Zealand	2007	SYBR green qPCR	7	0	S.Melzer	S.Shaw	negative	-37.35357	175.75848	No
StephanieShaw	236	Leiopelma archeyi	unknown	KomataReefs	Waikato	New Zealand	2007	SYBR green qPCR	5	0	S.Melzer	S.Shaw	negative	-37.35042	175.75730	No
StephanieShaw	237	Litoria aurea	female	ThamesKauaeranga Valley	Waikato	New Zealand	2007	SYBR green qPCR	1	0	S.Melzer	S.Shaw	negative	-37.13526	175.60529	No
StephanieShaw	238	Litoria aurea	male	ThamesKauaeranga Valley	Waikato	New Zealand	2007	SYBR green qPCR	3	0	S.Melzer	S.Shaw	negative	-37.13526	175.60529	No
StephanieShaw	239	Leiopelma archeyi	unknown	Tapu	Waikato	New Zealand	2007	SYBR green qPCR	5	0	S.Melzer	S.Shaw	negative	-36.98988	175.58861	No
StephanieShaw	240	Leiopelma hochstetteri	unknown	Tapu	Waikato	New Zealand	2007	SYBR green qPCR	10	0	S.Melzer	S.Shaw	negative	-36.98988	175.58861	No
StephanieShaw	241	Leiopelma hochstetteri	unknown	Tokatea	Waikato	New Zealand	2007	SYBR green qPCR	1	0	S.Melzer	S.Shaw	negative	-36.72652	175.52066	No
StephanieShaw	242	Leiopelma archeyi	unknown	Tokatea	Waikato	New Zealand	2007	SYBR green qPCR	3	0	S.Melzer	S.Shaw	negative	-36.72838	175.52129	No
StephanieShaw	243	Leiopelma hochstetteri	unknown	Tokatea	Waikato	New Zealand	2007	SYBR green qPCR	1	0	S.Melzer	S.Shaw	negative	-36.72838	175.52129	No
StephanieShaw	244	Leiopelma archeyi	unknown	MoehauPahi	Waikato	New Zealand	2007	SYBR green qPCR	8	0	S.Melzer	S.Shaw	negative	-36.52485	175.36443	No
StephanieShaw	245	Leiopelma hochstetteri	unknown	Hunua	Auckland	New Zealand	2007	SYBR green qPCR	4	0	S.Melzer	S.Shaw	negative	-37.01623	175.14485	No
StephanieShaw	246	Litoria aurea	male	KerikeriRangitane	Northland	New Zealand	2007	SYBR green qPCR	8	0	S.Melzer	S.Shaw	negative	-35.19038	173.98978	No
StephanieShaw	247	Litoria aurea	unknown	KerikeriCharles	Northland	New Zealand	2007	SYBR green qPCR	2	0	S.Melzer	S.Shaw	negative	-35.24733	173.90500	No
StephanieShaw	248	Leiopelma hochstetteri	unknown	Warkworth	Auckland	New Zealand	2007	SYBR green qPCR	6	0	S.Melzer	S.Shaw	negative	-36.33262	174.61690	No
StephanieShaw	249	Leiopelma hochstetteri	unknown	Warkworth	Auckland	New Zealand	2007	SYBR green qPCR	3	0	S.Melzer	S.Shaw	negative	-36.33763	174.63052	No
StephanieShaw	250	Litoria aurea	male	Tauronga	Bay of Plenty	New Zealand	2007	SYBR green qPCR	1	0	S.Melzer	S.Shaw	negative	-37.73456	176.11882	No
StephanieShaw	251	Litoria aurea	female	Tauronga	Bay of Plenty	New Zealand	2007	SYBR green qPCR	1	0	S.Melzer	S.Shaw	negative	-37.73456	176.11882	No
StephanieShaw	252	Litoria aurea	unknown	Taupo	Waikato	New Zealand	2007	SYBR green qPCR	3	0	S.Melzer	S.Shaw	negative	-38.67183	176.06475	No

StephanieShaw	253	Leiopelma hochstetteri	unknown	Opotiki	Bay of Plenty	New Zealand	2007	SYBR green qPCR	1	0	S.Melzer	S.Shaw	negative	-38.14247	177.44595	No
StephanieShaw	254	Litoria aurea	female	Opotiki	Bay of Plenty	New Zealand	2007	SYBR green qPCR	1	0	S.Melzer	S.Shaw	negative	-38.01192	177.17850	No
StephanieShaw	255	Leiopelma hochstetteri	unknown	Toatoa	Bay of Plenty	New Zealand	2007	SYBR green qPCR	1	0	S.Melzer	S.Shaw	negative	-38.16688	177.50163	No
StephanieShaw	256	Leiopelma hochstetteri	unknown	Toatoa	Bay of Plenty	New Zealand	2007	SYBR green qPCR	2	0	S.Melzer	S.Shaw	negative	-38.17175	177.49757	No
StephanieShaw	257	Litoria raniformis	male	Awakeri	Bay of Plenty	New Zealand	2007	SYBR green qPCR	1	0	S.Melzer	S.Shaw	negative	-38.04392	176.95632	No
StephanieShaw	258	Litoria raniformis	unknown	Awakeri	Bay of Plenty	New Zealand	2007	SYBR green qPCR	1	0	S.Melzer	S.Shaw	negative	-38.04392	176.95632	No
StephanieShaw	259	Litoria raniformis	male	Whangara	Gisborne	New Zealand	2007	SYBR green qPCR	8	0	S.Melzer	S.Shaw	negative	-38.48983	178.16053	No
StephanieShaw	260	Litoria raniformis	female	Whangara	Gisborne	New Zealand	2007	SYBR green qPCR	2	0	S.Melzer	S.Shaw	negative	-38.48983	178.16053	No
StephanieShaw	261	Litoria raniformis	unknown	Whangara	Gisborne	New Zealand	2007	SYBR green qPCR	1	0	S.Melzer	S.Shaw	negative	-38.48983	178.16053	No
StephanieShaw	262	Litoria raniformis	female	BayView	Hawkes Bay	New Zealand	2007	SYBR green qPCR	2	0	S.Melzer	S.Shaw	negative	-39.40270	176.85095	No
StephanieShaw	263	Litoria raniformis	male	BayView	Hawkes Bay	New Zealand	2007	SYBR green qPCR	3	0	S.Melzer	S.Shaw	negative	-39.40270	176.85095	No
StephanieShaw	264	Litoria ewingii	male	Wellington Wainuomata	Wellington	New Zealand	2007	SYBR green qPCR	4	0	S.Melzer	S.Shaw	negative	-41.26564	174.93360	No
StephanieShaw	265	Litoria ewingii	unknown	Wellington Wainuomata	Wellington	New Zealand	2007	SYBR green qPCR	1	0	S.Melzer	S.Shaw	negative	-41.26564	174.93360	No
StephanieShaw	266	Leiopelma pakeka	unknown	MaudIsland	Marlborough	New Zealand	2007	SYBR green qPCR	19	0	S.Melzer	S.Shaw	negative	-41.02247	173.89558	No
StephanieShaw	267	Leiopelma pakeka	unknown	MaudIsland	Marlborough	New Zealand	2005	TqPCR	30	0	B.Bell	S.Shaw	negative	-41.02447	173.89288	No
StephanieShaw	268	Leiopelma pakeka	unknown	MaudIsland	Marlborough	New Zealand	2006	TqPCR	30	0	B.Bell	S.Shaw	negative	-41.02447	173.89288	No
StephanieShaw	269	Leiopelma pakeka	unknown	MaudIsland	Marlborough	New Zealand	2008	TqPCR	60	0	B.Bell	S.Shaw	negative	-41.02447	173.89288	No
StephanieShaw	270	Leiopelma hochstetteri	unknown	Pukeamaru Raukumera	Gisborne	New Zealand	2009	TqPCR	20	0	M.Ohmer	S.Shaw	negative	-37.64822	178.24021	No
StephanieShaw	271	Litoria ewingii	male	Macraes	Otago	New Zealand	2009	TqPCR	14	14	M.Ohmer	S.Shaw	positive	-45.366667	170.416667	No
StephanieShaw	272	Litoria ewingii	unknown	Macraes	Otago	New Zealand	2009	TqPCR	2	2	M.Ohmer	S.Shaw	positive	-45.366667	170.416667	No
StephanieShaw	273	Litoria ewingii	female	Macraes	Otago	New Zealand	2009	TqPCR	4	4	M.Ohmer	S.Shaw	positive	-45.366667	170.416667	No
StephanieShaw	274	Leiopelma pakeka	unknown	MaudIsland	Marlborough	New Zealand	2009	TqPCR	5	0	P.Gaze	S.Shaw	negative	-41.016667	173.883333	No
StephanieShaw	275	Litoria ewingii	unknown	Winton	Southland	New Zealand	2010	TqPCR	2	0	M.Ohmer	S.Shaw	negative	-46.133333	168.333333	No
StephanieShaw	276	Litoria ewingii	unknown	Naseby	Otago	New Zealand	2010	TqPCR	7	0	M.Ohmer	S.Shaw	negative	-45.016667	170.166667	No
StephanieShaw	277	Litoria ewingii	unknown	OrokonuiWaitati	Otago	New Zealand	2010	TqPCR	2	1	M.Ohmer	S.Shaw	positive	-45.776728	170.605454	Dead
StephanieShaw	278	Leiopelma hamiltoni	unknown	StephensIsland	Marlborough	New Zealand	2009	TqPCR	80	0	P.Bishop	S.Shaw	negative	-40.66667	174	No

StephanieShaw	279	Litoria ewingii	unknown	ChathamIslands	Chatham Islands	New Zealand	2006	TqPCR	20	0	P.Bishop	S.Shaw	negative	-43.914218	-176.52729	no
StephanieShaw	280	Leiopelma archeyi	unknown	TapuBell	Waikato	New Zealand	2006	TqPCR	14	2	B.Bell	S.Shaw	positive	-36.9867	175.5814	No
StephanieShaw	281	Leiopelma hochstetteri	unknown	TapuBell	Waikato	New Zealand	2006	TqPCR	1	0	B.Bell	S.Shaw	negative	-36.9867	175.5814	No
StephanieShaw	282	Litoria aurea	unknown	HamiltonZoo	Waikato	New Zealand	2006	TqPCR	6	1	M.Crossland	S.Shaw	positive	-37.77415	175.21717	No
StephanieShaw	283	Leiopelma archeyi	unknown	Whareorino	Waikato	New Zealand	2001	TqPCR	1	0	M.Crossland	S.Shaw	negative	-38.4016	174.7950	Dead
StephanieShaw	284	Leiopelma archeyi	unknown	Whareorino	Waikato	New Zealand	2005	TqPCR	25	0	M.Crossland	S.Shaw	negative	-38.4065	174.7985	No
StephanieShaw	285	Leiopelma archeyi	unknown	Whareorino	Waikato	New Zealand	2005	TqPCR	6	0	M.Crossland	S.Shaw	negative	-38.4060	174.7972	No
StephanieShaw	286	Leiopelma archeyi	unknown	Whareorino	Waikato	New Zealand	2005	TqPCR	9	0	M.Crossland	S.Shaw	negative	-38.4063	174.7995	No
StephanieShaw	287	Leiopelma archeyi	unknown	TapuCoroglen Rd	Waikato	New Zealand	2001	TqPCR	1	1	M.Crossland	S.Shaw	positive	-36.97707	175.6049	No
StephanieShaw	288	Leiopelma archeyi	unknown	Moehau	Waikato	New Zealand	2001	TqPCR	1	1	M.Crossland	S.Shaw	positive	-36.54	175.4025	Dead
StephanieShaw	289	Leiopelma archeyi	unknown	TapuCoroglen Rd	Waikato	New Zealand	2002	TqPCR	1	1	M.Crossland	S.Shaw	positive	-36.97707	175.6049	Dead
StephanieShaw	290	Leiopelma archeyi	unknown	Whareorino	Waikato	New Zealand	2002	TqPCR	8	0	J.Wallace	S.Shaw	negative	-38.4016	174.7950	Dead
StephanieShaw	291	Leiopelma archeyi	unknown	Whareorino	Waikato	New Zealand	2003	TqPCR	8	0	J.Wallace	S.Shaw	negative	-38.4016	174.7950	Dead
StephanieShaw	292	Leiopelma archeyi	unknown	Whareorino	Waikato	New Zealand	2000	TqPCR	1	0	J.Wallace	S.Shaw	negative	-38.4016	174.7950	Dead
StephanieShaw	293	Leiopelma archeyi	unknown	Whareorino	Waikato	New Zealand	2001	TqPCR	3	0	J.Wallace	S.Shaw	negative	-38.4016	174.7950	Dead
StephanieShaw	294	Leiopelma archeyi	unknown	Whareorino	Waikato	New Zealand	2002	TqPCR	11	0	J.Wallace	S.Shaw	negative	-38.4016	174.7950	Dead
StephanieShaw	295	Leiopelma archeyi	unknown	Whareorino	Waikato	New Zealand	2003	TqPCR	1	0	J.Wallace	S.Shaw	negative	-38.4016	174.7950	Dead
StephanieShaw	296	Leiopelma archeyi	unknown	Whareorino	Waikato	New Zealand	2006	TqPCR	100	11	A.Haigh	S.Shaw	positive	-38.4016	174.7950	No
StephanieShaw	297	Leiopelma pakeka	unknown	MaudIsland	Marlborough	New Zealand	2006	TqPCR	35	0	A.Haigh	S.Shaw	negative	-41.016667	173.883333	No
StephanieShaw	298	Leiopelma archeyi	unknown	Whareorino	Waikato	New Zealand	2006	TqPCR	17	3	L.Daglish	S.Shaw	positive	-38.4063	174.7995	No
StephanieShaw	299	Leiopelma archeyi	unknown	Whareorino	Waikato	New Zealand	2006	TqPCR	7	1	L.Daglish	S.Shaw	positive	-38.4065	174.7985	No
StephanieShaw	300	Leiopelma archeyi	unknown	Whareorino	Waikato	New Zealand	2007	TqPCR	4	1	L.Daglish	S.Shaw	positive	-38.4065	174.7985	No
StephanieShaw	301	Leiopelma archeyi	unknown	Whareorino	Waikato	New Zealand	2006	TqPCR	43	1	L.Daglish	S.Shaw	negative	-38.3802	174.7940	No
StephanieShaw	302	Leiopelma archeyi	unknown	Whareorino	Waikato	New Zealand	2007	TqPCR	15	1	L.Daglish	S.Shaw	positive	-38.3802	174.7940	No
StephanieShaw	303	Leiopelma archeyi	unknown	Whareorino	Waikato	New Zealand	2006	TqPCR	57	4	L.Daglish	S.Shaw	positive	-38.3801	174.7930	No
StephanieShaw	304	Leiopelma archeyi	unknown	Whareorino	Waikato	New Zealand	2007	TqPCR	9	0	L.Daglish	S.Shaw	negative	-38.3801	174.7930	No

StephanieShaw	305	Leiopelma archeyi	unknown	TapuBell	Waikato	New Zealand	2007	TqPCR	12	0	B.Bell	S.Shaw	negative	-36.9867	175.5814	No
StephanieShaw	306	Leiopelma archeyi	unknown	MoehauPahigrid	Waikato	New Zealand	2007	TqPCR	6	0	A.Haigh	S.Shaw	negative	-36.524694	175.365566	No
StephanieShaw	307	Leiopelma archeyi	unknown	Pukeokahu	Waikato	New Zealand	2007	TqPCR	4	0	L.Daglish	S.Shaw	negative	-38.4034	175.532859	No
StephanieShaw	308	Leiopelma hochstetteri	unknown	Mareretu	Northland	New Zealand	2008	TqPCR	1	0	A.Haigh	S.Shaw	negative	-36.043187	174.413016	No
StephanieShaw	309	Leiopelma hochstetteri	unknown	Mareretu	Northland	New Zealand	2008	TqPCR	1	0	A.Haigh	S.Shaw	negative	-36.014484	174.376299	No
StephanieShaw	310	Leiopelma hochstetteri	unknown	Mareretu	Northland	New Zealand	2008	TqPCR	1	0	A.Haigh	S.Shaw	negative	-36.014291	174.375884	No
StephanieShaw	311	Leiopelma hochstetteri	unknown	Mareretu	Northland	New Zealand	2008	TqPCR	1	0	A.Haigh	S.Shaw	negative	-36.014121	174.375814	No
StephanieShaw	312	Leiopelma hochstetteri	unknown	Mareretu	Northland	New Zealand	2008	TqPCR	1	0	A.Haigh	S.Shaw	negative	-36.014291	174.375884	No
StephanieShaw	313	Leiopelma hochstetteri	unknown	Mareretu	Northland	New Zealand	2008	TqPCR	1	0	A.Haigh	S.Shaw	negative	-36.014398	174.375997	No
StephanieShaw	314	Leiopelma hochstetteri	unknown	Mareretu	Northland	New Zealand	2008	TqPCR	1	0	A.Haigh	S.Shaw	negative	-36.01377	174.380437	No
StephanieShaw	315	Leiopelma hochstetteri	unknown	Mareretu	Northland	New Zealand	2008	TqPCR	2	0	A.Haigh	S.Shaw	negative	-36.013299	174.375953	No
StephanieShaw	316	Leiopelma hochstetteri	unknown	Mareretu	Northland	New Zealand	2008	TqPCR	1	0	A.Haigh	S.Shaw	negative	-36.013171	174.375418	No
StephanieShaw	317	Leiopelma hochstetteri	unknown	Brynderwyn	Northland	New Zealand	2008	TqPCR	1	0	A.Haigh	S.Shaw	negative	-36.078476	174.422521	No
StephanieShaw	318	Leiopelma hochstetteri	unknown	Brynderwyn	Northland	New Zealand	2008	TqPCR	2	0	A.Haigh	S.Shaw	negative	-36.078514	174.42241	No
StephanieShaw	319	Leiopelma hochstetteri	unknown	Brynderwyn	Northland	New Zealand	2008	TqPCR	1	0	A.Haigh	S.Shaw	negative	-36.078621	174.42249	No
StephanieShaw	320	Leiopelma hochstetteri	unknown	Brynderwyn	Northland	New Zealand	2008	TqPCR	1	0	A.Haigh	S.Shaw	negative	-36.078376	174.422574	No
StephanieShaw	321	Leiopelma hochstetteri	unknown	Brynderwyn	Northland	New Zealand	2008	TqPCR	1	0	A.Haigh	S.Shaw	negative	-36.078739	174.422426	No
StephanieShaw	322	Leiopelma hochstetteri	unknown	Brynderwyn	Northland	New Zealand	2008	TqPCR	2	0	A.Haigh	S.Shaw	negative	-36.078421	174.422553	No
StephanieShaw	323	Leiopelma hochstetteri	unknown	Brynderwyn	Northland	New Zealand	2008	TqPCR	1	0	A.Haigh	S.Shaw	negative	-36.078222	174.422626	No
StephanieShaw	324	Leiopelma hochstetteri	unknown	Brynderwyn	Northland	New Zealand	2008	TqPCR	1	0	A.Haigh	S.Shaw	negative	-36.078295	174.422595	No
StephanieShaw	325	Leiopelma hochstetteri	unknown	Brynderwyn	Northland	New Zealand	2008	TqPCR	3	0	A.Haigh	S.Shaw	negative	-36.078306	174.422439	No
StephanieShaw	326	Leiopelma hochstetteri	unknown	Brynderwyn	Northland	New Zealand	2008	TqPCR	1	0	A.Haigh	S.Shaw	negative	-36.078667	174.422414	No
StephanieShaw	327	Leiopelma hochstetteri	unknown	Brynderwyn	Northland	New Zealand	2008	TqPCR	1	0	A.Haigh	S.Shaw	negative	-36.078695	174.422336	No
StephanieShaw	328	Leiopelma hochstetteri	unknown	Brynderwyn	Northland	New Zealand	2008	TqPCR	1	0	A.Haigh	S.Shaw	negative	-36.078911	174.42233	No
StephanieShaw	329	Leiopelma hochstetteri	unknown	Brynderwyn	Northland	New Zealand	2008	TqPCR	2	0	A.Haigh	S.Shaw	negative	-36.077981	174.422477	No
StephanieShaw	330	Leiopelma hochstetteri	unknown	Brynderwyn	Northland	New Zealand	2008	TqPCR	2	0	A.Haigh	S.Shaw	negative	-36.07753	174.422501	No

StephanieShaw	331	Leiopelma hochstetteri	unknown	TotaraPeak	Auckland	New Zealand	2008	TqPCR	1	0	A.Haigh	S.Shaw	negative	-36.327044	174.662785	No
StephanieShaw	332	Leiopelma hochstetteri	unknown	TotaraPeak	Auckland	New Zealand	2008	TqPCR	1	0	A.Haigh	S.Shaw	negative	-36.327736	174.664651	No
StephanieShaw	333	Leiopelma hochstetteri	unknown	TotaraPeak	Auckland	New Zealand	2008	TqPCR	1	0	A.Haigh	S.Shaw	negative	-36.327345	174.664842	No
StephanieShaw	334	Leiopelma hochstetteri	unknown	TotaraPeak	Auckland	New Zealand	2008	TqPCR	1	0	A.Haigh	S.Shaw	negative	-36.327048	174.665926	No
StephanieShaw	335	Leiopelma hochstetteri	unknown	Dome	Auckland	New Zealand	2008	TqPCR	3	0	A.Haigh	S.Shaw	negative	-36.339377	174.652844	No
StephanieShaw	336	Leiopelma hochstetteri	unknown	Dome	Auckland	New Zealand	2008	TqPCR	1	0	A.Haigh	S.Shaw	negative	-36.33926	174.652217	No
StephanieShaw	337	Leiopelma hochstetteri	unknown	Dome	Auckland	New Zealand	2008	TqPCR	2	0	A.Haigh	S.Shaw	negative	-36.339279	174.652195	No
StephanieShaw	338	Leiopelma hochstetteri	unknown	Dome	Auckland	New Zealand	2008	TqPCR	4	0	A.Haigh	S.Shaw	negative	-36.339186	174.651781	No
StephanieShaw	339	Leiopelma hochstetteri	unknown	Dome	Auckland	New Zealand	2008	TqPCR	2	0	A.Haigh	S.Shaw	negative	-36.33927	174.651594	No
StephanieShaw	340	Leiopelma hochstetteri	unknown	Dome	Auckland	New Zealand	2008	TqPCR	1	0	A.Haigh	S.Shaw	negative	-36.339176	174.651257	No
StephanieShaw	341	Leiopelma hochstetteri	unknown	Dome	Auckland	New Zealand	2008	TqPCR	1	0	A.Haigh	S.Shaw	negative	-36.339189	174.650979	No
StephanieShaw	342	Leiopelma hochstetteri	unknown	Dome	Auckland	New Zealand	2008	TqPCR	4	0	A.Haigh	S.Shaw	negative	-36.338974	174.650896	No
StephanieShaw	343	Leiopelma hochstetteri	unknown	Dome	Auckland	New Zealand	2008	TqPCR	2	0	A.Haigh	S.Shaw	negative	-36.33898	174.651141	No
StephanieShaw	344	Leiopelma hochstetteri	unknown	Dome	Auckland	New Zealand	2008	TqPCR	1	0	A.Haigh	S.Shaw	negative	-36.339048	174.631292	No
StephanieShaw	345	Leiopelma hochstetteri	unknown	Dome	Auckland	New Zealand	2008	TqPCR	1	0	A.Haigh	S.Shaw	negative	-36.340875	174.631289	No
StephanieShaw	346	Leiopelma hochstetteri	unknown	Dome	Auckland	New Zealand	2008	TqPCR	1	0	A.Haigh	S.Shaw	negative	-36.340206	174.631176	No
StephanieShaw	347	Leiopelma hochstetteri	unknown	Dome	Auckland	New Zealand	2008	TqPCR	1	0	A.Haigh	S.Shaw	negative	-36.329311	174.605497	No
StephanieShaw	348	Leiopelma hochstetteri	unknown	Dome	Auckland	New Zealand	2008	TqPCR	1	0	A.Haigh	S.Shaw	negative	-36.329411	174.60538	No
StephanieShaw	349	Leiopelma hochstetteri	unknown	Dome	Auckland	New Zealand	2008	TqPCR	1	0	A.Haigh	S.Shaw	negative	-36.329434	174.6057	No
StephanieShaw	350	Leiopelma hochstetteri	unknown	Dome	Auckland	New Zealand	2008	TqPCR	2	0	A.Haigh	S.Shaw	negative	-36.329464	174.605534	No
StephanieShaw	351	Leiopelma hochstetteri	unknown	HunuaKMA	Auckland	New Zealand	2008	TqPCR	3	0	A.Haigh	S.Shaw	negative	-37.040191	175.195264	No
StephanieShaw	352	Leiopelma hochstetteri	unknown	HunuaKMA	Auckland	New Zealand	2008	TqPCR	1	0	A.Haigh	S.Shaw	negative	-37.040082	175.195294	No
StephanieShaw	353	Leiopelma hochstetteri	unknown	HunuaKMA	Auckland	New Zealand	2008	TqPCR	1	0	A.Haigh	S.Shaw	negative	-37.039346	175.195563	No
StephanieShaw	354	Leiopelma hochstetteri	unknown	HunuaKMA	Auckland	New Zealand	2008	TqPCR	8	0	A.Haigh	S.Shaw	negative	-37.041161	175.197633	No
StephanieShaw	355	Leiopelma hochstetteri	unknown	HunuaKMA	Auckland	New Zealand	2008	TqPCR	3	0	A.Haigh	S.Shaw	negative	-37.047368	175.196039	No
StephanieShaw	356	Leiopelma hochstetteri	unknown	HunuaKMA	Auckland	New Zealand	2008	TqPCR	2	0	A.Haigh	S.Shaw	negative	-37.046293	175.195514	No

StephanieShaw	357	Leiopelma hochstetteri	unknown	HunuaKMA	Auckland	New Zealand	2008	TqPCR	2	0	A.Haigh	S.Shaw	negative	-37.047073	175.19549	No
StephanieShaw	358	Leiopelma hochstetteri	unknown	HunuaKMA	Auckland	New Zealand	2008	TqPCR	5	0	A.Haigh	S.Shaw	negative	-37.046903	175.195417	No
StephanieShaw	359	Leiopelma hochstetteri	unknown	HunuaKMA	Auckland	New Zealand	2008	TqPCR	1	0	A.Haigh	S.Shaw	negative	-37.046741	175.195423	No
StephanieShaw	360	Leiopelma hochstetteri	unknown	HunuaKMA	Auckland	New Zealand	2008	TqPCR	2	0	A.Haigh	S.Shaw	negative	-37.046465	175.195224	No
StephanieShaw	361	Leiopelma hochstetteri	unknown	HunuaKMA	Auckland	New Zealand	2008	TqPCR	1	0	A.Haigh	S.Shaw	negative	-37.046317	175.195421	No
StephanieShaw	362	Leiopelma hochstetteri	unknown	HunuaKMA	Auckland	New Zealand	2008	TqPCR	4	0	A.Haigh	S.Shaw	negative	-37.045441	175.195034	No
StephanieShaw	363	Leiopelma hochstetteri	unknown	HunuaKMA	Auckland	New Zealand	2008	TqPCR	1	0	A.Haigh	S.Shaw	negative	-37.043678	175.203829	No
StephanieShaw	364	Leiopelma hochstetteri	unknown	HunuaKMA	Auckland	New Zealand	2008	TqPCR	1	0	A.Haigh	S.Shaw	negative	-37.04391	175.203938	No
StephanieShaw	365	Leiopelma hochstetteri	unknown	HunuaKMA	Auckland	New Zealand	2008	TqPCR	3	0	A.Haigh	S.Shaw	negative	-37.043902	175.203904	No
StephanieShaw	366	Leiopelma hochstetteri	unknown	HunuaKMA	Auckland	New Zealand	2008	TqPCR	3	0	A.Haigh	S.Shaw	negative	-37.044175	175.203755	No
StephanieShaw	367	Leiopelma hochstetteri	unknown	HunuaKMA	Auckland	New Zealand	2008	TqPCR	4	0	A.Haigh	S.Shaw	negative	-37.044046	175.203886	No
StephanieShaw	368	Leiopelma hochstetteri	unknown	HunuaKMA	Auckland	New Zealand	2008	TqPCR	4	0	A.Haigh	S.Shaw	negative	-37.125884	175.207548	No
StephanieShaw	369	Leiopelma hochstetteri	unknown	HunuaKMA	Auckland	New Zealand	2008	TqPCR	2	0	A.Haigh	S.Shaw	negative	-37.044387	175.203975	No
StephanieShaw	370	Leiopelma hochstetteri	unknown	HunuaKMA	Auckland	New Zealand	2008	TqPCR	1	0	A.Haigh	S.Shaw	negative	-37.044596	175.203914	No
StephanieShaw	371	Leiopelma hochstetteri	unknown	HunuaKMA	Auckland	New Zealand	2008	TqPCR	1	0	A.Haigh	S.Shaw	negative	-37.044711	175.203996	No
StephanieShaw	372	Leiopelma hochstetteri	unknown	PahiStream SEMoehau	Waikato	New Zealand	2008	TqPCR	2	0	A.Haigh	S.Shaw	negative	-36.524916	175.364903	No
StephanieShaw	373	Leiopelma hochstetteri	unknown	PahiStream SEMoehau	Waikato	New Zealand	2008	TqPCR	1	0	A.Haigh	S.Shaw	negative	-36.525004	175.365855	No
StephanieShaw	374	Leiopelma hochstetteri	unknown	PahiStream SEMoehau	Waikato	New Zealand	2008	TqPCR	1	0	A.Haigh	S.Shaw	negative	-36.524501	175.366598	No
StephanieShaw	375	Leiopelma hochstetteri	unknown	PahiStream SEMoehau	Waikato	New Zealand	2008	TqPCR	2	0	A.Haigh	S.Shaw	negative	-36.52464	175.366815	No
StephanieShaw	376	Leiopelma hochstetteri	unknown	PahiStream SEMoehau	Waikato	New Zealand	2008	TqPCR	1	0	A.Haigh	S.Shaw	negative	-36.525098	175.365691	No
StephanieShaw	377	Leiopelma hochstetteri	unknown	PahiStream SEMoehau	Waikato	New Zealand	2008	TqPCR	4	0	A.Haigh	S.Shaw	negative	-36.525244	175.365584	No
StephanieShaw	378	Leiopelma hochstetteri	unknown	PahiStream SEMoehau	Waikato	New Zealand	2008	TqPCR	2	0	A.Haigh	S.Shaw	negative	-36.524995	175.367072	No
StephanieShaw	379	Leiopelma hochstetteri	unknown	StonyBay Moehau	Waikato	New Zealand	2008	TqPCR	1	0	A.Haigh	S.Shaw	negative	-36.511357	175.433901	No
StephanieShaw	380	Leiopelma hochstetteri	unknown	StonyBay Moehau	Waikato	New Zealand	2008	TqPCR	7	0	A.Haigh	S.Shaw	negative	-36.502518	175.414789	No
StephanieShaw	381	Leiopelma hochstetteri	unknown	StonyBay Moehau	Waikato	New Zealand	2008	TqPCR	2	0	A.Haigh	S.Shaw	negative	-36.502553	175.415109	No
StephanieShaw	382	Leiopelma hochstetteri	unknown	StonyBaY Moehau	Waikato	New Zealand	2008	TqPCR	3	0	A.Haigh	S.Shaw	negative	-36.502563	175.414618	No

StephanieShaw	383	Leiopelma hochstetteri	unknown	StonyBay Moehau	Waikato	New Zealand	2008	TqPCR	1	0	A.Haigh	S.Shaw	negative	-36.502596	175.415189	No
StephanieShaw	384	Leiopelma hochstetteri	unknown	StonyBayMoehau	Waikato	New Zealand	2008	TqPCR	2	0	A.Haigh	S.Shaw	negative	-36.50254	175.415276	No
StephanieShaw	385	Leiopelma hochstetteri	unknown	StonyBayMoehau	Waikato	New Zealand	2008	TqPCR	1	0	A.Haigh	S.Shaw	negative	-36.500202	175.414583	No
StephanieShaw	386	Leiopelma hochstetteri	unknown	StonyBayMoehau	Waikato	New Zealand	2008	TqPCR	1	0	A.Haigh	S.Shaw	negative	-36.50316	175.415755	No
StephanieShaw	387	Leiopelma hochstetteri	unknown	PortCharlesRd	Waikato	New Zealand	2008	TqPCR	4	0	A.Haigh	S.Shaw	negative	-36.560713	175.472877	No
StephanieShaw	388	Leiopelma hochstetteri	unknown	PortCharlesRd	Waikato	New Zealand	2008	TqPCR	1	0	A.Haigh	S.Shaw	negative	-36.551749	175.470449	No
StephanieShaw	389	Leiopelma hochstetteri	unknown	309Rd	Waikato	New Zealand	2008	TqPCR	1	0	A.Haigh	S.Shaw	negative	-36.838904	175.55382	No
StephanieShaw	390	Leiopelma hochstetteri	unknown	309Rd	Waikato	New Zealand	2008	TqPCR	1	0	A.Haigh	S.Shaw	negative	-36.839101	175.553906	No
StephanieShaw	391	Leiopelma hochstetteri	unknown	309Rd	Waikato	New Zealand	2008	TqPCR	2	0	A.Haigh	S.Shaw	negative	-36.839082	175.553961	No
StephanieShaw	392	Leiopelma hochstetteri	unknown	309Rd	Waikato	New Zealand	2008	TqPCR	2	0	A.Haigh	S.Shaw	negative	-36.839041	175.554162	No
StephanieShaw	393	Leiopelma hochstetteri	unknown	309Rd	Waikato	New Zealand	2008	TqPCR	1	0	A.Haigh	S.Shaw	negative	-36.839527	175.554594	No
StephanieShaw	394	Leiopelma hochstetteri	unknown	309Rd	Waikato	New Zealand	2008	TqPCR	1	0	A.Haigh	S.Shaw	negative	-36.839805	175.554638	No
StephanieShaw	395	Leiopelma hochstetteri	unknown	309Rd	Waikato	New Zealand	2008	TqPCR	1	0	A.Haigh	S.Shaw	negative	-36.840059	175.554961	No
StephanieShaw	396	Leiopelma hochstetteri	unknown	309Rd	Waikato	New Zealand	2008	TqPCR	1	0	A.Haigh	S.Shaw	negative	-36.840036	175.554803	No
StephanieShaw	397	Leiopelma hochstetteri	unknown	309Rd	Waikato	New Zealand	2008	TqPCR	1	0	A.Haigh	S.Shaw	negative	-36.848482	175.559896	No
StephanieShaw	398	Leiopelma hochstetteri	unknown	309Rd	Waikato	New Zealand	2008	TqPCR	2	0	A.Haigh	S.Shaw	negative	-36.848128	175.560007	No
StephanieShaw	399	Leiopelma hochstetteri	unknown	309Rd	Waikato	New Zealand	2008	TqPCR	5	0	A.Haigh	S.Shaw	negative	-36.848774	175.560479	No
StephanieShaw	400	Leiopelma hochstetteri	unknown	309Rd	Waikato	New Zealand	2008	TqPCR	1	0	A.Haigh	S.Shaw	negative	-36.848329	175.560642	No
StephanieShaw	401	Leiopelma hochstetteri	unknown	309Rd	Waikato	New Zealand	2008	TqPCR	2	0	A.Haigh	S.Shaw	negative	-36.848085	175.560678	No
StephanieShaw	402	Leiopelma hochstetteri	unknown	309Rd	Waikato	New Zealand	2008	TqPCR	1	0	A.Haigh	S.Shaw	negative	-36.858834	175.548108	No
StephanieShaw	403	Leiopelma hochstetteri	unknown	309Rd	Waikato	New Zealand	2008	TqPCR	2	0	A.Haigh	S.Shaw	negative	-36.858806	175.548129	No
StephanieShaw	404	Leiopelma hochstetteri	unknown	309Rd	Waikato	New Zealand	2008	TqPCR	1	0	A.Haigh	S.Shaw	negative	-36.859104	175.547321	No
StephanieShaw	405	Leiopelma hochstetteri	unknown	309Rd	Waikato	New Zealand	2008	TqPCR	1	0	A.Haigh	S.Shaw	negative	-36.859075	175.547028	No
StephanieShaw	406	Leiopelma hochstetteri	unknown	309Rd	Waikato	New Zealand	2008	TqPCR	1	0	A.Haigh	S.Shaw	negative	-36.859168	175.546908	No
StephanieShaw	407	Leiopelma hochstetteri	unknown	309Rd	Waikato	New Zealand	2008	TqPCR	4	0	A.Haigh	S.Shaw	negative	-36.858735	175.546152	No
StephanieShaw	408	Leiopelma hochstetteri	unknown	TapuCoroglen Rd	Waikato	New Zealand	2008	TqPCR	2	0	A.Haigh	S.Shaw	negative	-36.991194	175.566292	No

StephanieShaw	409	Leiopelma hochstetteri	unknown	TapuCoroglen Rd	Waikato	New Zealand	2008	TqPCR	2	0	A.Haigh	S.Shaw	negative	-36.991279	175.566542	No
StephanieShaw	410	Leiopelma hochstetteri	unknown	TapuCoroglen Rd	Waikato	New Zealand	2008	TqPCR	1	0	A.Haigh	S.Shaw	negative	-36.991325	175.566499	No
StephanieShaw	411	Leiopelma hochstetteri	unknown	TapuCoroglen Rd	Waikato	New Zealand	2008	TqPCR	1	0	A.Haigh	S.Shaw	negative	-36.991167	175.565909	No
StephanieShaw	412	Leiopelma hochstetteri	unknown	TapuCoroglen Rd	Waikato	New Zealand	2008	TqPCR	1	0	A.Haigh	S.Shaw	negative	-36.991171	175.566111	No
StephanieShaw	413	Leiopelma hochstetteri	unknown	TapuCoroglen Rd	Waikato	New Zealand	2008	TqPCR	4	0	A.Haigh	S.Shaw	negative	-36.989862	175.571998	No
StephanieShaw	414	Leiopelma hochstetteri	unknown	TapuCoroglen Rd	Waikato	New Zealand	2008	TqPCR	1	0	A.Haigh	S.Shaw	negative	-36.990264	175.572181	No
StephanieShaw	415	Leiopelma hochstetteri	unknown	TapuCoroglen Rd	Waikato	New Zealand	2008	TqPCR	5	0	A.Haigh	S.Shaw	negative	-36.990555	175.572057	No
StephanieShaw	416	Leiopelma hochstetteri	unknown	TapuCoroglen Rd	Waikato	New Zealand	2008	TqPCR	2	0	A.Haigh	S.Shaw	negative	-36.991779	175.579867	No
StephanieShaw	417	Leiopelma hochstetteri	unknown	TapuCoroglen Rd	Waikato	New Zealand	2008	TqPCR	4	0	A.Haigh	S.Shaw	negative	-36.991689	175.579852	No
StephanieShaw	418	Leiopelma hochstetteri	unknown	TapuCoroglen Rd	Waikato	New Zealand	2008	TqPCR	2	0	A.Haigh	S.Shaw	negative	-36.99187	175.579825	No
StephanieShaw	419	Leiopelma hochstetteri	unknown	TapuCoroglen Rd	Waikato	New Zealand	2008	TqPCR	1	0	A.Haigh	S.Shaw	negative	-36.983789	175.592264	No
StephanieShaw	420	Leiopelma hochstetteri	unknown	TapuCoroglen Rd	Waikato	New Zealand	2008	TqPCR	5	0	A.Haigh	S.Shaw	negative	-36.983682	175.592226	No
StephanieShaw	421	Leiopelma hochstetteri	unknown	25aRoadKopu HikuaiRd	Waikato	New Zealand	2008	TqPCR	2	0	A.Haigh	S.Shaw	negative	-37.146389	175.67101	No
StephanieShaw	422	Leiopelma hochstetteri	unknown	BrokenHills	Waikato	New Zealand	2008	TqPCR	2	0	A.Haigh	S.Shaw	negative	-37.110201	175.736029	No
StephanieShaw	423	Leiopelma hochstetteri	unknown	BrokenHills	Waikato	New Zealand	2008	TqPCR	2	0	A.Haigh	S.Shaw	negative	-37.111157	175.73566	No
StephanieShaw	424	Leiopelma hochstetteri	unknown	BrokenHills	Waikato	New Zealand	2008	TqPCR	3	0	A.Haigh	S.Shaw	negative	-37.111268	175.735563	No
StephanieShaw	425	Leiopelma hochstetteri	unknown	BrokenHills	Waikato	New Zealand	2008	TqPCR	1	0	A.Haigh	S.Shaw	negative	-37.111242	175.735517	No
StephanieShaw	426	Leiopelma hochstetteri	unknown	BrokenHills	Waikato	New Zealand	2008	TqPCR	1	0	A.Haigh	S.Shaw	negative	-37.111429	175.727489	No
StephanieShaw	427	Leiopelma hochstetteri	unknown	BrokenHills	Waikato	New Zealand	2008	TqPCR	1	0	A.Haigh	S.Shaw	negative	-37.111457	175.73521	No
StephanieShaw	428	Leiopelma hochstetteri	unknown	BrokenHills	Waikato	New Zealand	2008	TqPCR	3	0	A.Haigh	S.Shaw	negative	-37.112359	175.727489	No
StephanieShaw	429	Leiopelma hochstetteri	unknown	GoldenCross	Waikato	New Zealand	2008	TqPCR	1	0	A.Haigh	S.Shaw	negative	-37.329885	175.77882	No
StephanieShaw	430	Leiopelma hochstetteri	unknown	GoldenCross	Waikato	New Zealand	2008	TqPCR	1	0	A.Haigh	S.Shaw	negative	-37.329268	175.778616	No
StephanieShaw	431	Leiopelma hochstetteri	unknown	GoldenCross	Waikato	New Zealand	2008	TqPCR	2	0	A.Haigh	S.Shaw	negative	-37.32917	175.778567	No
StephanieShaw	432	Leiopelma hochstetteri	unknown	GoldenCross	Waikato	New Zealand	2008	TqPCR	3	0	A.Haigh	S.Shaw	negative	-37.329085	175.77836	No
StephanieShaw	433	Leiopelma hochstetteri	unknown	GoldenCross	Waikato	New Zealand	2008	TqPCR	3	0	A.Haigh	S.Shaw	negative	-37.328902	175.778116	No
StephanieShaw	434	Leiopelma hochstetteri	unknown	GoldenCross	Waikato	New Zealand	2008	TqPCR	1	0	A.Haigh	S.Shaw	negative	-37.328504	175.778157	No

StephanieShaw	435	Leiopelma hochstetteri	unknown	GoldenCross	Waikato	New Zealand	2008	TqPCR	1	0	A.Haigh	S.Shaw	negative	-37.328877	175.778024	No
StephanieShaw	436	Leiopelma hochstetteri	unknown	GoldenCross	Waikato	New Zealand	2008	TqPCR	1	0	A.Haigh	S.Shaw	negative	-37.32883	175.778124	No
StephanieShaw	437	Leiopelma hochstetteri	unknown	GoldenCross	Waikato	New Zealand	2008	TqPCR	2	0	A.Haigh	S.Shaw	negative	-37.328855	175.777459	No
StephanieShaw	438	Leiopelma hochstetteri	unknown	GoldenCross	Waikato	New Zealand	2008	TqPCR	2	0	A.Haigh	S.Shaw	negative	-37.323009	175.779804	No
StephanieShaw	439	Leiopelma hochstetteri	unknown	GoldenCross	Waikato	New Zealand	2008	TqPCR	2	0	A.Haigh	S.Shaw	negative	-37.320785	175.778238	No
StephanieShaw	440	Leiopelma hochstetteri	unknown	GoldenCross	Waikato	New Zealand	2008	TqPCR	1	0	A.Haigh	S.Shaw	negative	-37.320823	175.778161	No
StephanieShaw	441	Leiopelma hochstetteri	unknown	GoldenCross	Waikato	New Zealand	2008	TqPCR	1	0	A.Haigh	S.Shaw	negative	-37.320626	175.778446	No
StephanieShaw	442	Leiopelma hochstetteri	unknown	GoldenCross	Waikato	New Zealand	2008	TqPCR	1	0	A.Haigh	S.Shaw	negative	-37.320689	175.778463	No
StephanieShaw	443	Leiopelma hochstetteri	unknown	GoldenCross	Waikato	New Zealand	2008	TqPCR	3	0	A.Haigh	S.Shaw	negative	-37.320589	175.778513	No
StephanieShaw	444	Leiopelma hochstetteri	unknown	GoldenCross	Waikato	New Zealand	2008	TqPCR	1	0	A.Haigh	S.Shaw	negative	-37.320823	175.778533	No
StephanieShaw	445	Leiopelma hochstetteri	unknown	GoldenCross	Waikato	New Zealand	2008	TqPCR	2	0	A.Haigh	S.Shaw	negative	-37.320339	175.778379	No
StephanieShaw	446	Leiopelma hochstetteri	unknown	GoldenCross	Waikato	New Zealand	2008	TqPCR	1	0	A.Haigh	S.Shaw	negative	-37.320358	175.778707	No
StephanieShaw	447	Leiopelma hochstetteri	unknown	GoldenCross	Waikato	New Zealand	2008	TqPCR	1	0	A.Haigh	S.Shaw	negative	-37.320379	175.778956	No
StephanieShaw	448	Leiopelma hochstetteri	unknown	GoldenCross	Waikato	New Zealand	2008	TqPCR	1	0	A.Haigh	S.Shaw	negative	-37.320317	175.77892	No
StephanieShaw	449	Leiopelma hochstetteri	unknown	KomataReefs	Waikato	New Zealand	2008	TqPCR	1	0	A.Haigh	S.Shaw	negative	-37.354938	175.739642	No
StephanieShaw	450	Leiopelma hochstetteri	unknown	KopuHikuai	Waikato	New Zealand	2008	TqPCR	1	0	A.Haigh	S.Shaw	negative	-37.070874	175.773838	Dead
StephanieShaw	451	Leiopelma hochstetteri	unknown	KopuHikuai	Waikato	New Zealand	2008	TqPCR	1	0	A.Haigh	S.Shaw	negative	-37.070874	175.773838	No
StephanieShaw	452	Leiopelma hochstetteri	unknown	TapuBell	Waikato	New Zealand	2008	TqPCR	5	0	A.Haigh	S.Shaw	negative	-36.9867	175.5814	No
StephanieShaw	453	Leiopelma hochstetteri	unknown	Otawa	Bay of Plenty	New Zealand	2008	TqPCR	1	0	A.Haigh	S.Shaw	negative	-37.825358	176.234077	No
StephanieShaw	454	Leiopelma hochstetteri	unknown	Otawa	Bay of Plenty	New Zealand	2008	TqPCR	1	0	A.Haigh	S.Shaw	negative	-37.824498	176.234208	No
StephanieShaw	455	Leiopelma hochstetteri	unknown	Otawa	Bay of Plenty	New Zealand	2008	TqPCR	1	0	A.Haigh	S.Shaw	negative	-37.823814	176.234472	No
StephanieShaw	456	Leiopelma hochstetteri	unknown	Otawa	Bay of Plenty	New Zealand	2008	TqPCR	3	0	A.Haigh	S.Shaw	negative	-37.823741	176.234503	No
StephanieShaw	457	Leiopelma hochstetteri	unknown	Otawa	Bay of Plenty	New Zealand	2008	TqPCR	1	0	A.Haigh	S.Shaw	negative	-37.823733	176.23448	No
StephanieShaw	458	Leiopelma hochstetteri	unknown	Otawa	Bay of Plenty	New Zealand	2008	TqPCR	3	0	A.Haigh	S.Shaw	negative	-37.82361	176.234667	No
StephanieShaw	459	Leiopelma hochstetteri	unknown	Otawa	Bay of Plenty	New Zealand	2008	TqPCR	1	0	A.Haigh	S.Shaw	negative	-37.823131	176.234714	No
StephanieShaw	460	Leiopelma hochstetteri	unknown	Otawa	Bay of Plenty	New Zealand	2008	TqPCR	3	0	A.Haigh	S.Shaw	negative	-37.823129	176.23477	No

StephanieShaw	461	Leiopelma hochstetteri	unknown	Otawa	Bay of Plenty	New Zealand	2008	TqPCR	2	0	A.Haigh	S.Shaw	negative	-37.823053	176.234915	No
StephanieShaw	462	Leiopelma hochstetteri	unknown	Otawa	Bay of Plenty	New Zealand	2008	TqPCR	1	0	A.Haigh	S.Shaw	negative	-37.822682	176.234966	No
StephanieShaw	463	Leiopelma hochstetteri	unknown	Otawa	Bay of Plenty	New Zealand	2008	TqPCR	3	0	A.Haigh	S.Shaw	negative	-37.822548	176.234937	No
StephanieShaw	464	Leiopelma hochstetteri	unknown	Otawa	Bay of Plenty	New Zealand	2008	TqPCR	2	0	A.Haigh	S.Shaw	negative	-37.822001	176.234833	No
StephanieShaw	465	Leiopelma hochstetteri	unknown	Otawa	Bay of Plenty	New Zealand	2008	TqPCR	1	0	A.Haigh	S.Shaw	negative	-37.821996	176.234991	No
StephanieShaw	466	Leiopelma hochstetteri	unknown	Otawa	Bay of Plenty	New Zealand	2008	TqPCR	2	0	A.Haigh	S.Shaw	negative	-37.818554	176.239597	No
StephanieShaw	467	Leiopelma hochstetteri	unknown	Otawa	Bay of Plenty	New Zealand	2008	TqPCR	1	0	A.Haigh	S.Shaw	negative	-37.824052	176.239678	No
StephanieShaw	468	Leiopelma hochstetteri	unknown	Pakihi	Bay of Plenty	New Zealand	2008	TqPCR	3	0	A.Haigh	S.Shaw	negative	-38.125863	177.389625	No
StephanieShaw	469	Leiopelma hochstetteri	unknown	Pakihi	Bay of Plenty	New Zealand	2008	TqPCR	3	0	A.Haigh	S.Shaw	negative	-38.128437	177.432364	No
StephanieShaw	470	Leiopelma hochstetteri	unknown	Pakihi	Bay of Plenty	New Zealand	2008	TqPCR	1	0	A.Haigh	S.Shaw	negative	-38.128339	177.432118	No
StephanieShaw	471	Leiopelma hochstetteri	unknown	Pakihi	Bay of Plenty	New Zealand	2008	TqPCR	1	0	A.Haigh	S.Shaw	negative	-38.128641	177.432	No
StephanieShaw	472	Leiopelma hochstetteri	unknown	Pakihi	Bay of Plenty	New Zealand	2008	TqPCR	1	0	A.Haigh	S.Shaw	negative	-38.12882	177.432023	No
StephanieShaw	473	Leiopelma hochstetteri	unknown	Pakihi	Bay of Plenty	New Zealand	2008	TqPCR	1	0	A.Haigh	S.Shaw	negative	-38.106203	177.395567	No
StephanieShaw	474	Leiopelma hochstetteri	unknown	Pakihi	Bay of Plenty	New Zealand	2008	TqPCR	1	0	A.Haigh	S.Shaw	negative	-38.106477	177.395732	No
StephanieShaw	475	Leiopelma hochstetteri	unknown	Pakihi	Bay of Plenty	New Zealand	2008	TqPCR	2	0	A.Haigh	S.Shaw	negative	-38.098078	177.373046	No
StephanieShaw	476	Leiopelma hochstetteri	unknown	Motu	Gisborne	New Zealand	2008	TqPCR	1	0	A.Haigh	S.Shaw	negative	-38.312951	177.505427	No
StephanieShaw	477	Leiopelma hochstetteri	unknown	Motu	Gisborne	New Zealand	2008	TqPCR	1	0	A.Haigh	S.Shaw	negative	-38.313596	177.506235	No
StephanieShaw	478	Leiopelma hochstetteri	unknown	Motu	Gisborne	New Zealand	2008	TqPCR	4	0	A.Haigh	S.Shaw	negative	-38.313563	177.506141	No
StephanieShaw	479	Leiopelma hochstetteri	unknown	Motu	Gisborne	New Zealand	2008	TqPCR	1	0	A.Haigh	S.Shaw	negative	-38.313053	177.505136	No
StephanieShaw	480	Leiopelma hochstetteri	unknown	Motu	Gisborne	New Zealand	2008	TqPCR	3	0	A.Haigh	S.Shaw	negative	-38.313062	177.504896	No
StephanieShaw	481	Leiopelma hochstetteri	unknown	Motu	Gisborne	New Zealand	2008	TqPCR	9	0	A.Haigh	S.Shaw	negative	-38.312858	177.504826	No
StephanieShaw	482	Leiopelma hochstetteri	unknown	Takaputahi	Bay of Plenty	New Zealand	2008	TqPCR	5	0	A.Haigh	S.Shaw	negative	-38.086223	177.475967	No
StephanieShaw	483	Leiopelma hochstetteri	unknown	Takaputahi	Bay of Plenty	New Zealand	2008	TqPCR	3	0	A.Haigh	S.Shaw	negative	-38.083239	177.578754	No
StephanieShaw	484	Leiopelma hochstetteri	unknown	Takaputahi	Bay of Plenty	New Zealand	2008	TqPCR	1	0	A.Haigh	S.Shaw	negative	-38.083446	177.57854	No
StephanieShaw	485	Leiopelma hochstetteri	unknown	Takaputahi	Bay of Plenty	New Zealand	2008	TqPCR	2	0	A.Haigh	S.Shaw	negative	-38.083261	177.578185	No
StephanieShaw	486	Leiopelma hochstetteri	unknown	Takaputahi	Bay of Plenty	New Zealand	2008	TqPCR	3	0	A.Haigh	S.Shaw	negative	-38.083504	177.578429	No

StephanieShaw	487	Leiopelma hochstetteri	unknown	Takaputahi	Bay of Plenty	New Zealand	2008	TqPCR	1	0	A.Haigh	S.Shaw	negative	-38.083269	177.577992	No
StephanieShaw	488	Leiopelma hochstetteri	unknown	WhanaruaBay	Bay of Plenty	New Zealand	2008	TqPCR	2	0	A.Haigh	S.Shaw	negative	-37.685408	177.788609	No
StephanieShaw	489	Leiopelma hochstetteri	unknown	WhanaruaBay	Bay of Plenty	New Zealand	2008	TqPCR	2	0	A.Haigh	S.Shaw	negative	-37.685535	177.789015	No
StephanieShaw	490	Leiopelma hochstetteri	unknown	WhanaruaBay	Bay of Plenty	New Zealand	2008	TqPCR	1	0	A.Haigh	S.Shaw	negative	-37.685454	177.789009	No
StephanieShaw	491	Leiopelma hochstetteri	unknown	WhanaruaBay	Bay of Plenty	New Zealand	2008	TqPCR	2	0	A.Haigh	S.Shaw	negative	-37.686178	177.786606	No
StephanieShaw	492	Leiopelma hochstetteri	unknown	WhanaruaBay	Bay of Plenty	New Zealand	2008	TqPCR	2	0	A.Haigh	S.Shaw	negative	-37.685993	177.786287	No
StephanieShaw	493	Leiopelma hochstetteri	unknown	WhanaruaBay	Bay of Plenty	New Zealand	2008	TqPCR	1	0	A.Haigh	S.Shaw	negative	-37.685885	177.785837	No
StephanieShaw	494	Leiopelma hochstetteri	unknown	WhanaruaBay	Bay of Plenty	New Zealand	2008	TqPCR	2	0	A.Haigh	S.Shaw	negative	-37.685629	177.785695	No
StephanieShaw	495	Leiopelma hochstetteri	unknown	WhanaruaBay	Bay of Plenty	New Zealand	2008	TqPCR	2	0	A.Haigh	S.Shaw	negative	-37.685501	177.785312	No
StephanieShaw	496	Leiopelma hochstetteri	unknown	WhanaruaBay	Bay of Plenty	New Zealand	2008	TqPCR	1	0	A.Haigh	S.Shaw	negative	-37.685426	177.785171	No
StephanieShaw	497	Leiopelma hochstetteri	unknown	WhanaruaBay	Bay of Plenty	New Zealand	2008	TqPCR	5	0	A.Haigh	S.Shaw	negative	-37.685482	177.785129	No
StephanieShaw	498	Leiopelma hochstetteri	unknown	Raukokore	Bay of Plenty	New Zealand	2008	TqPCR	2	0	A.Haigh	S.Shaw	negative	-37.67926	177.868123	No
StephanieShaw	499	Leiopelma hochstetteri	unknown	Raukokore	Bay of Plenty	New Zealand	2008	TqPCR	2	0	A.Haigh	S.Shaw	negative	-37.679484	177.867934	No
StephanieShaw	500	Leiopelma hochstetteri	unknown	Raukokore	Bay of Plenty	New Zealand	2008	TqPCR	2	0	A.Haigh	S.Shaw	negative	-37.679487	177.867662	No
StephanieShaw	501	Leiopelma hochstetteri	unknown	Raukokore	Bay of Plenty	New Zealand	2008	TqPCR	5	0	A.Haigh	S.Shaw	negative	-37.679637	177.867467	No
StephanieShaw	502	Leiopelma hochstetteri	unknown	Raukokore	Bay of Plenty	New Zealand	2008	TqPCR	3	0	A.Haigh	S.Shaw	negative	-37.679283	177.867148	No
StephanieShaw	503	Leiopelma hochstetteri	unknown	PukeamaruRaukumera	Gisborne	New Zealand	2008	TqPCR	9	0	A.Haigh	S.Shaw	negative	-37.65548	178.242382	No
StephanieShaw	504	Leiopelma hochstetteri	unknown	LeitchsHut	Waikato	New Zealand	2008	TqPCR	1	0	A.Haigh	S.Shaw	negative	-38.411883	174.799993	No
StephanieShaw	505	Leiopelma hochstetteri	unknown	LeitchsHut	Waikato	New Zealand	2008	TqPCR	2	0	A.Haigh	S.Shaw	negative	-38.414558	174.796764	No
StephanieShaw	506	Leiopelma hochstetteri	unknown	LeitchsHut	Waikato	New Zealand	2008	TqPCR	1	0	A.Haigh	S.Shaw	negative	-38.414664	174.795759	No
StephanieShaw	507	Leiopelma hochstetteri	unknown	LeitchsHut	Waikato	New Zealand	2008	TqPCR	2	0	A.Haigh	S.Shaw	negative	-38.41847	174.790063	No
StephanieShaw	508	Leiopelma hochstetteri	unknown	LeitchsHut	Waikato	New Zealand	2008	TqPCR	1	0	A.Haigh	S.Shaw	negative	-38.418329	174.789876	No
StephanieShaw	509	Leiopelma hochstetteri	unknown	RangitotoPureora	Waikato	New Zealand	2008	TqPCR	1	0	A.Haigh	S.Shaw	negative	-38.363171	175.522887	No
StephanieShaw	510	Leiopelma hochstetteri	unknown	RangitotoPureora	Waikato	New Zealand	2008	TqPCR	1	0	A.Haigh	S.Shaw	negative	-38.363751	175.523949	No
StephanieShaw	511	Leiopelma archeyi	unknown	TapuBell	Waikato	New Zealand	2007	TqPCR	10	3	B.Bell	S.Shaw	positive	-36.9867	175.5814	No
StephanieShaw	512	Leiopelma archeyi	unknown	MoehauPahigrid	Waikato	New Zealand	2007	TqPCR	3	0	A.Haigh	S.Shaw	negative	-36.524694	175.365566	No

StephanieShaw	513	Leiopelma archeyi	unknown	TeHope	Waikato	New Zealand	2007	TqPCR	2	0	A.Haigh	S.Shaw	negative	-36.546191	175.420761	No
StephanieShaw	514	Leiopelma archeyi	unknown	KomataReefs	Waikato	New Zealand	2007	TqPCR	3	0	A.Haigh	S.Shaw	negative	-37.348901	175.755907	No
StephanieShaw	515	Leiopelma hochstetteri	unknown	SquareKauriStream	Waikato	New Zealand	2008	TqPCR	3	0	A.Haigh	S.Shaw	negative	-36.981541	175.533414	No
StephanieShaw	516	Litoria ewingii	unknown	GooseBay	Canterbury	New Zealand	2008	TqPCR	6	0	S.Shaw	S.Shaw	negative	-42.478197	173.52915	Dead
StephanieShaw	517	Leiopelma hochstetteri	unknown	RangitotoPureora	Waikato	New Zealand	2009	TqPCR	26	0	A.Haigh	S.Shaw	negative	-38.363171	175.522887	No
StephanieShaw	518	Leiopelma hochstetteri	unknown	Whangapoua	Waikato	New Zealand	2009	TqPCR	1	0	A.Haigh	S.Shaw	negative	-36.713534	175.598001	Dead
StephanieShaw	519	Leiopelma hochstetteri	unknown	HunuaRanges	Auckland	New Zealand	2008	TqPCR	1	0	A.Haigh	S.Shaw	negative	-37.0691	175.2266	Dead
StephanieShaw	520	Leiopelma hochstetteri	unknown	Maungatautari	Waikato	New Zealand	2009	TqPCR	5	0	A.Haigh	S.Shaw	negative	-38.004347	175.566587	No
StephanieShaw	521	Leiopelma hochstetteri	unknown	KaimaiRanges	Waikato	New Zealand	2009	TqPCR	29	0	A.Haigh	S.Shaw	negative	-37.786453	175.954285	No
StephanieShaw	522	Leiopelma hochstetteri	unknown	Brynderwyn	Waikato	New Zealand	2009	TqPCR	4	0	A.Haigh	S.Shaw	negative	-36.07753	174.422501	No
StephanieShaw	523	Leiopelma archeyi	unknown	KomataReefs	Waikato	New Zealand	2009	TqPCR	1	0	A.Haigh	S.Shaw	negative	-37.348901	175.755907	No
StephanieShaw	524	Leiopelma archeyi	unknown	MoehauPahigrid	Waikato	New Zealand	2009	TqPCR	3	1	A.Haigh	S.Shaw	positive	-36.524694	175.365566	No
StephanieShaw	525	Leiopelma archeyi	unknown	TapuBell	Waikato	New Zealand	2008	TqPCR	18	3	B.Bell	S.Shaw	positive	-36.9867	175.5814	No
StephanieShaw	526	Leiopelma archeyi	unknown	Whareorino	Waikato	New Zealand	2008	TqPCR	9	0	L.Daglish	S.Shaw	negative	-38.4063	174.7995	No
StephanieShaw	527	Leiopelma archeyi	unknown	Whareorino	Waikato	New Zealand	2009	TqPCR	2	0	L.Daglish	S.Shaw	negative	-38.4063	174.7995	No
StephanieShaw	528	Leiopelma archeyi	unknown	Whareorino	Waikato	New Zealand	2007	TqPCR	13	0	L.Daglish	S.Shaw	negative	-38.4065	174.7985	No
StephanieShaw	529	Leiopelma archeyi	unknown	Whareorino	Waikato	New Zealand	2008	TqPCR	28	0	L.Daglish	S.Shaw	negative	-38.4065	174.7985	No
StephanieShaw	530	Leiopelma archeyi	unknown	Whareorino	Waikato	New Zealand	2009	TqPCR	14	0	L.Daglish	S.Shaw	negative	-38.4065	174.7985	No
StephanieShaw	531	Leiopelma archeyi	unknown	Whareorino	Waikato	New Zealand	2008	TqPCR	9	0	L.Daglish	S.Shaw	negative	-38.3802	174.7940	No
StephanieShaw	532	Leiopelma archeyi	unknown	Whareorino	Waikato	New Zealand	2007	TqPCR	26	0	L.Daglish	S.Shaw	negative	-38.3802	174.7940	No
StephanieShaw	533	Leiopelma archeyi	unknown	Whareorino	Waikato	New Zealand	2007	TqPCR	45	2	L.Daglish	S.Shaw	positive	-38.3801	174.7930	No
StephanieShaw	534	Leiopelma archeyi	unknown	Whareorino	Waikato	New Zealand	2008	TqPCR	27	2	L.Daglish	S.Shaw	positive	-38.3801	174.7930	No
StephanieShaw	535	Leiopelma archeyi	unknown	Whareorino	Waikato	New Zealand	2009	TqPCR	32	0	L.Daglish	S.Shaw	negative	-38.3801	174.7930	No
StephanieShaw	536	Leiopelma archeyi	unknown	Pukeokahu	Waikato	New Zealand	2008	TqPCR	1	0	L.Daglish	S.Shaw	negative	-38.4034	175.532859	No
StephanieShaw	537	Leiopelma archeyi	unknown	Pukeokahu	Waikato	New Zealand	2008	TqPCR	1	0	L.Daglish	S.Shaw	negative	-38.4034	175.532859	Dead
StephanieShaw	538	Litoria ewingii	male	MerivaleOtautau	Southland	New Zealand	1951	histology	2	0	unknown	S.Shaw	negative	-46.122176	167.9910051	unknown

StephanieShaw	539	Litoria raniformis	unknown	LakeRotoiti	Tasman	New Zealand	1939	histology	2	0	S.Shaw	S.Shaw	negative	-41.820374	172.837135	unknown
StephanieShaw	540	Litoria raniformis	male	Mangere	Auckland	New Zealand	1982	histology	1	0	S.Shaw	S.Shaw	negative	-36.997311	174.76439	unknown
StephanieShaw	541	Litoria raniformis	unknown	Mangere	Auckland	New Zealand	1982	histology	2	0	S.Shaw	S.Shaw	negative	-36.968628	174.793687	unknown
StephanieShaw	542	Litoria aurea	unknown	MtWellington	Auckland	New Zealand	1982	histology	1	0	S.Shaw	S.Shaw	negative	-36.908485	174.83874	unknown
StephanieShaw	543	Litoria aurea	unknown	Auckland	Auckland	New Zealand	1981	histology	1	0	S.Shaw	S.Shaw	negative	-36.84846	174.763332	unknown
StephanieShaw	544	Litoria aurea	unknown	Auckland	Auckland	New Zealand	1985	histology	2	0	S.Shaw	S.Shaw	negative	-36.84846	174.763332	unknown
StephanieShaw	545	Litoria aurea	female	Kawerua	Northland	New Zealand	1983	histology	1	0	S.Shaw	S.Shaw	negative	-35.625956	173.4674	unknown
StephanieShaw	546	Litoria aurea	female	MtWellington	Auckland	New Zealand	1983	histology	1	0	S.Shaw	S.Shaw	negative	-36.908485	174.83874	unknown
StephanieShaw	547	Litoria aurea	female	Piha	Auckland	New Zealand	1985	histology	1	0	S.Shaw	S.Shaw	negative	-36.953021	174.468809	unknown
StephanieShaw	548	Litoria aurea	male	Kaipara	Northland	New Zealand	1988	histology	1	0	S.Shaw	S.Shaw	negative	-36.363799	174.163513	unknown
StephanieShaw	549	Litoria aurea	female	Whitford	Auckland	New Zealand	1991	histology	1	0	S.Shaw	S.Shaw	negative	-36.945628	174.963688	unknown
StephanieShaw	550	Litoria aurea	unknown	Ahipara	Northland	New Zealand	1984	histology	1	0	S.Shaw	S.Shaw	negative	-35.171339	173.153272	unknown
StephanieShaw	551	Litoria aurea	unknown	RiverheadKumeu	Auckland	New Zealand	1999	histology	1	0	S.Shaw	S.Shaw	negative	-36.73668	174.5918459	unknown
StephanieShaw	552	Leiopelma archeyi	unknown	Tokatea	Waikato	New Zealand	1983	histology	1	0	S.Shaw	S.Shaw	negative	-36.736683	174.568634	unknown
StephanieShaw	553	Leiopelma archeyi	unknown	Tokatea	Waikato	New Zealand	1981	histology	2	0	S.Shaw	S.Shaw	negative	-36.691823	175.5337143	unknown
StephanieShaw	554	Leiopelma archeyi	unknown	Coromandel	Waikato	New Zealand	1930	histology	2	0	S.Shaw	S.Shaw	negative	-36.691823	175.5337143	unknown
StephanieShaw	555	Leiopelma archeyi	unknown	Coromandel	Waikato	New Zealand	1983	histology	1	0	S.Shaw	S.Shaw	negative	-36.691823	175.5337143	unknown
StephanieShaw	556	Leiopelma archeyi	unknown	Whareorino	Waikato	New Zealand	1981	histology	1	0	S.Shaw	S.Shaw	negative	-38.416667	174.6833333	unknown
StephanieShaw	557	Leiopelma hochstetteri	unknown	Coromandel	Waikato	New Zealand	1958	histology	1	0	S.Shaw	S.Shaw	negative	-36.945628	174.963688	unknown
StephanieShaw	558	Leiopelma hochstetteri	unknown	TeAraroa	Waikato	New Zealand	1937	histology	2	0	S.Shaw	S.Shaw	negative	-37.620835	178.3692001	unknown
StephanieShaw	559	Leiopelma hochstetteri	unknown	HuiaWaitakere	Waikato	New Zealand	1936	histology	2	0	S.Shaw	S.Shaw	negative	-36.987099	174.5649941	unknown
StephanieShaw	560	Leiopelma hochstetteri	female	HuiaWaitakere	Waikato	New Zealand	1936	histology	3	0	S.Shaw	S.Shaw	negative	-36.987099	174.5649941	unknown
StephanieShaw	561	Leiopelma hochstetteri	unknown	Warkworth	Auckland	New Zealand	1955	histology	1	0	S.Shaw	S.Shaw	negative	-36.398687	174.660509	unknown
StephanieShaw	562	Leiopelma hochstetteri	unknown	Tokatea	Waikato	New Zealand	1938	histology	2	0	S.Shaw	S.Shaw	negative	-36.691823	175.5337143	unknown
StephanieShaw	563	Leiopelma hochstetteri	unknown	TeAraroa	Waikato	New Zealand	1982	histology	3	0	S.Shaw	S.Shaw	negative	-37.635208	178.3692	unknown
StephanieShaw	564	Leiopelma hochstetteri	unknown	Warkworth	Waikato	New Zealand	1941	histology	1	0	S.Shaw	S.Shaw	negative	-36.398687	174.660509	unknown

StephanieShaw	565	Leiopelma hochstetteri	unknown	MtTeAroha	Waikato	New Zealand	1982	histology	1	0	S.Shaw	S.Shaw	negative	-37.542591	175.711731	unknown
StephanieShaw	566	Leiopelma hochstetteri	unknown	Whareorino	Waikato	New Zealand	1996	histology	1	0	S.Shaw	S.Shaw	negative	-38.416667	174.6833333	unknown
StephanieShaw	567	Leiopelma hochstetteri	female	Whareorino	Waikato	New Zealand	1998	histology	1	0	S.Shaw	S.Shaw	negative	-38.416667	174.683333	unknown
StephanieShaw	568	Leiopelma hochstetteri	unknown	Waitakere	Auckland	New Zealand	1998	histology	1	0	S.Shaw	S.Shaw	negative	-36.910647	174.5440006	unknown
StephanieShaw	569	Leiopelma hochstetteri	unknown	Waitakere	Auckland	New Zealand	1995	histology	1	0	P.Bishop	S.Shaw	negative	-36.910647	174.5440006	unknown
StephanieShaw	570	Litoria ewingii	male	KarangaruaRiver	West Coast	New Zealand	1985	histology	1	0	P.Bishop	S.Shaw	negative	-43.534363	169.8056316	unknown
StephanieShaw	571	Litoria ewingii	male	CookRiverS	West Coast	New Zealand	1986	histology	1	0	P.Bishop	S.Shaw	negative	-43.437714	169.7967911	unknown
StephanieShaw	572	Litoria ewingii	female	CookRiverS	West Coast	New Zealand	1986	histology	5	0	P.Bishop	S.Shaw	negative	-43.437714	169.7967911	unknown
StephanieShaw	573	Litoria ewingii	unknown	CookRiverS	West Coast	New Zealand	1986	histology	9	0	P.Bishop	S.Shaw	negative	-43.437714	169.7967911	unknown
StephanieShaw	574	Leiopelma hochstetteri	unknown	TeAraroa	Gisborne	New Zealand	1939	histology	5	0	S.Shaw	S.Shaw	negative	-37.635208	178.3692	unknown
StephanieShaw	575	Leiopelma hochstetteri	unknown	Kopuapounamu River	Gisborne	New Zealand	1950	histology	5	0	S.Shaw	S.Shaw	negative	-37.695981	178.3199069	unknown
StephanieShaw	576	Leiopelma hochstetteri	female	Karekare	Auckland	New Zealand	1954	histology	1	0	S.Shaw	S.Shaw	negative	-36.986602	174.479668	unknown
StephanieShaw	577	Leiopelma hochstetteri	unknown	Coromandel	Waikato	New Zealand	1972	histology	1	0	S.Shaw	S.Shaw	negative	-36.738884	175.534058	unknown
StephanieShaw	578	Leiopelma hochstetteri	unknown	CoromandelRange	Waikato	New Zealand	1971	histology	1	0	S.Shaw	S.Shaw	negative	-36.973758	175.602035	unknown
StephanieShaw	579	Leiopelma hochstetteri	unknown	KotorepupuaiStream	Waikato	New Zealand	1972	histology	1	0	S.Shaw	S.Shaw	negative	-37.192938	175.6601429	unknown
StephanieShaw	580	Leiopelma hochstetteri	unknown	Waipu	Northland	New Zealand	1972	histology	1	0	S.Shaw	S.Shaw	negative	-35.984731	174.4471371	unknown
StephanieShaw	581	Leiopelma hochstetteri	unknown	MangakakarikiStrmAwatereRiver	Gisborne	New Zealand	1939	histology	1	0	S.Shaw	S.Shaw	negative	-37.629324	178.3662987	unknown
StephanieShaw	582	Leiopelma hochstetteri	unknown	MangatutaraRiver	Gisborne	New Zealand	1971	histology	1	0	S.Shaw	S.Shaw	negative	-37.93331	177.819443	unknown
StephanieShaw	583	Leiopelma hochstetteri	unknown	Whakatane	Bay of Plenty	New Zealand	1971	histology	1	0	S.Shaw	S.Shaw	negative	-37.965019	176.974325	unknown
StephanieShaw	584	Leiopelma hochstetteri	unknown	TokateaRidge	Waikato	New Zealand	1973	histology	1	0	S.Shaw	S.Shaw	negative	-36.691823	175.5337143	unknown
StephanieShaw	585	Leiopelma hochstetteri	unknown	Whangamata	Waikato	New Zealand	1965	histology	1	0	S.Shaw	S.Shaw	negative	-37.207279	175.871152	unknown
StephanieShaw	586	Leiopelma hochstetteri	unknown	CoromandelRange	Waikato	New Zealand	1973	histology	1	0	S.Shaw	S.Shaw	negative	-36.672825	175.5127716	unknown
StephanieShaw	587	Leiopelma hochstetteri	unknown	CoromandelRange	Waikato	New Zealand	1974	histology	2	0	S.Shaw	S.Shaw	negative	-36.672825	175.5127716	unknown
StephanieShaw	588	Litoria raniformis	unknown	Wainuiomata	Waikato	New Zealand	1949	histology	5	0	S.Shaw	S.Shaw	negative	-41.263362	174.947905	unknown
StephanieShaw	589	Litoria ewingii	unknown	Greymouth	West Coast	New Zealand	1949	histology	2	0	S.Shaw	S.Shaw	negative	-42.454498	171.206478	unknown

StephanieShaw	590	Litoria raniformis	unknown	Ward Island	Wellington	New Zealand	1935	histology	1	0	S.Shaw	S.Shaw	negative	-41.293947	174.871614	unknown
StephanieShaw	591	Litoria raniformis	female	ConnellyRdLowerHutt	Wellington	New Zealand	1952	histology	1	0	S.Shaw	S.Shaw	negative	-41.203982	174.9117175	unknown
StephanieShaw	592	Litoria raniformis	unknown	NgatokotokoRiverTaupo	Waikato	New Zealand	1953	histology	1	0	S.Shaw	S.Shaw	negative	-38.761044	175.7256317	unknown
StephanieShaw	593	Litoria raniformis	female	SpaHotelTaupo	Waikato	New Zealand	1956	histology	1	0	S.Shaw	S.Shaw	negative	-38.693951	176.0664653	unknown
StephanieShaw	594	Litoria raniformis	male	SpaHotelTaupo	Waikato	New Zealand	1956	histology	1	0	S.Shaw	S.Shaw	negative	-38.693951	176.0664653	unknown
StephanieShaw	595	Litoria aurea	unknown	MayorIsland	Bay of Plenty	New Zealand	1956	histology	2	0	S.Shaw	S.Shaw	negative	-37.286159	176.2514126	unknown
StephanieShaw	596	Litoria raniformis	unknown	ManawatuRivermouth	Manawatu	New Zealand	1961	histology	1	0	S.Shaw	S.Shaw	negative	-40.466801	175.2188873	unknown
StephanieShaw	597	Litoria raniformis	unknown	Punapawa	Nelson	New Zealand	1955	histology	1	0	S.Shaw	S.Shaw	negative	-40.670223	172.398834	unknown
StephanieShaw	598	Litoria ewingii	unknown	Foxton	Manawatu	New Zealand	1963	histology	1	0	S.Shaw	S.Shaw	negative	-40.4725	175.285779	unknown
StephanieShaw	599	Litoria ewingii	unknown	Kowhitirangi	West Coast	New Zealand	1969	histology	3	0	S.Shaw	S.Shaw	negative	-42.877554	171.014256	unknown
StephanieShaw	600	Litoria ewingii	unknown	StokesValley	Wellington	New Zealand	1977	histology	1	0	S.Shaw	S.Shaw	negative	-41.174458	174.982015	unknown
StephanieShaw	601	Litoria raniformis	unknown	WaioekaGorge	Bay of Plenty	New Zealand	1966	histology	1	0	S.Shaw	S.Shaw	negative	-38.090526	177.288094	unknown
StephanieShaw	602	Litoria raniformis	unknown	MataiValley	Nelson	New Zealand	1991	histology	1	0	S.Shaw	S.Shaw	negative	-41.291222	173.323746	unknown
StephanieShaw	603	Litoria raniformis	unknown	Waitarere	Manawatu	New Zealand	1965	histology	1	0	S.Shaw	S.Shaw	negative	-40.55529	175.215172	unknown
StephanieShaw	604	Litoria ewingii	unknown	TarnLewisPass	West Coast	New Zealand	1967	histology	1	0	S.Shaw	S.Shaw	negative	-42.387459	172.384415	unknown
StephanieShaw	605	Litoria ewingii	unknown	BoyleLewisRiverjxn	Canterbury	New Zealand	1969	histology	8	0	S.Shaw	S.Shaw	negative	-42.555468	172.394627	unknown
StephanieShaw	606	Litoria ewingii	unknown	Hanmer	Canterbury	New Zealand	1971	histology	2	0	S.Shaw	S.Shaw	negative	-42.504503	172.694092	unknown
StephanieShaw	607	Litoria ewingii	unknown	Waitarere	Manawatu	New Zealand	1971	histology	1	0	S.Shaw	S.Shaw	negative	-40.55529	175.215172	unknown
StephanieShaw	608	Litoria raniformis	unknown	Wellington	Wellington	New Zealand	1976	histology	1	0	S.Shaw	S.Shaw	negative	-41.28646	174.776236	unknown
StephanieShaw	609	Litoria raniformis	unknown	HoughtonBay	Wellington	New Zealand	1976	histology	1	0	S.Shaw	S.Shaw	negative	-41.34434	174.789906	unknown
StephanieShaw	610	Litoria ewingii	unknown	Waitarere	Manawatu	New Zealand	1968	histology	1	0	S.Shaw	S.Shaw	negative	-40.55529	175.215172	unknown
StephanieShaw	611	Litoria raniformis	unknown	OretiBeach	Southland	New Zealand	1997	histology	1	0	S.Shaw	S.Shaw	negative	-46.438249	168.231821	unknown
StephanieShaw	612	Litoria raniformis	unknown	Riverton	Southland	New Zealand	1990	histology	1	0	S.Shaw	S.Shaw	negative	-46.363881	168.018195	unknown
StephanieShaw	613	Litoria aurea	unknown	Auckland	Auckland	New Zealand	1998	histology	1	0	S.Shaw	S.Shaw	negative	-36.849715	174.767547	unknown
StephanieShaw	614	Litoria raniformis	unknown	Taita	Wellington	New Zealand	1969	histology	3	0	A.Haigh	S.Shaw	negative	-41.179824	174.960771	unknown
StephanieShaw	615	Leiopelma archeyi	female	StonyBayMoehau	Waikato	New Zealand	2005	TqPCR	1	0	S.Shaw	S.Shaw	negative	-36.528472	175.418982	Dead

StephanieShaw	616	Leiopelma archeyi	unknown	StonyBayMoehau	Waikato	New Zealand	2005	TqPCR	1	0	S.Shaw	S.Shaw	negative	-36.524214	175.42202	Dead
StephanieShaw	617	Leiopelma archeyi	male	StonyBayMoehau	Waikato	New Zealand	2005	TqPCR	1	0	S.Shaw	S.Shaw	negative	-36.528528	175.418135	Dead
StephanieShaw	618	Leiopelma archeyi	unknown	StonyBayMoehau	Waikato	New Zealand	2004	TqPCR	2	0	S.Shaw	S.Shaw	negative	-36.528432	175.418399	Dead
StephanieShaw	619	Leiopelma archeyi	unknown	StonyBayMoehau	Waikato	New Zealand	2005	TqPCR	2	0	S.Shaw	S.Shaw	negative	-36.528534	175.417856	Dead
StephanieShaw	620	Leiopelma archeyi	unknown	StonyBayMoehau	Waikato	New Zealand	2006	TqPCR	1	0	S.Shaw	S.Shaw	negative	-36.528495	175.418424	Dead
StephanieShaw	621	Leiopelma archeyi	unknown	StonyBayMoehau	Waikato	New Zealand	2006	TqPCR	1	0	S.Shaw	S.Shaw	negative	-36.528567	175.418002	Dead
StephanieShaw	622	Leiopelma archeyi	unknown	StonyBayMoehau	Waikato	New Zealand	2003	TqPCR	1	0	S.Shaw	S.Shaw	negative	-36.514205	175.398485	Dead
StephanieShaw	623	Leiopelma archeyi	female	StonyBayMoehau	Waikato	New Zealand	2003	TqPCR	1	0	S.Shaw	S.Shaw	negative	-36.523765	175.422764	Dead
StephanieShaw	624	Leiopelma archeyi	male	StonyBayMoehau	Waikato	New Zealand	2004	TqPCR	1	0	S.Shaw	S.Shaw	negative	-36.528411	175.418968	Dead
StephanieShaw	625	Leiopelma archeyi	unknown	StonyBayMoehau	Waikato	New Zealand	2004	TqPCR	1	0	S.Shaw	S.Shaw	negative	-36.524279	175.421933	Dead
StephanieShaw	626	Leiopelma archeyi	unknown	StonyBayMoehau	Waikato	New Zealand	2004	TqPCR	1	0	S.Shaw	S.Shaw	negative	-36.524244	175.421887	Dead
StephanieShaw	627	Leiopelma archeyi	male	StonyBayMoehau	Waikato	New Zealand	2004	TqPCR	1	0	S.Shaw	S.Shaw	negative	-36.528534	175.418303	Dead
StephanieShaw	628	Leiopelma archeyi	female	StonyBayMoehau	Waikato	New Zealand	2004	TqPCR	1	0	S.Shaw	S.Shaw	negative	-36.528591	175.41817	Dead
StephanieShaw	629	Leiopelma archeyi	unknown	StonyBayMoehau	Waikato	New Zealand	2004	TqPCR	1	0	S.Shaw	S.Shaw	negative	-36.528534	175.418303	Dead
StephanieShaw	630	Leiopelma hochstetteri	female	StonyBayMoehau	Waikato	New Zealand	2006	TqPCR	1	0	S.Shaw	S.Shaw	negative	-36.528432	175.418399	Dead
StephanieShaw	631	Leiopelma hochstetteri	male	StonyBayMoehau	Waikato	New Zealand	2002	TqPCR	1	0	S.Shaw	S.Shaw	negative	-37.125884	175.207548	Dead
StephanieShaw	632	Leiopelma hochstetteri	unknown	StonyBayMoehau	Waikato	New Zealand	2003	TqPCR	1	0	S.Shaw	S.Shaw	negative	-37.125884	175.207548	Dead
StephanieShaw	633	Leiopelma hochstetteri	male	StonyBayMoehau	Waikato	New Zealand	2004	TqPCR	1	0	S.Shaw	S.Shaw	negative	-38.044306	175.55706	Dead
StephanieShaw	634	Leiopelma hochstetteri	male	StonyBayMoehau	Waikato	New Zealand	2004	TqPCR	1	0	S.Shaw	S.Shaw	negative	-36.528495	175.418424	Dead
StephanieShaw	635	Leiopelma archeyi	male	StonyBayMoehau	Waikato	New Zealand	2004	TqPCR	1	0	S.Shaw	S.Shaw	negative	-36.528474	175.418982	Dead
StephanieShaw	636	Leiopelma archeyi	male	StonyBayMoehau	Waikato	New Zealand	2005	TqPCR	1	0	S.Shaw	S.Shaw	negative	-36.528481	175.418256	Dead
StephanieShaw	637	Leiopelma hochstetteri	male	StonyBayMoehau	Waikato	New Zealand	2005	TqPCR	1	0	S.Shaw	S.Shaw	negative	-37.125884	175.207548	Dead
StephanieShaw	638	Leiopelma archeyi	male	StonyBayMoehau	Waikato	New Zealand	2006	TqPCR	1	0	S.Shaw	S.Shaw	negative	-36.524008	175.422415	Dead
StephanieShaw	639	Leiopelma archeyi	unknown	StonyBayMoehau	Waikato	New Zealand	2006	TqPCR	1	0	S.Shaw	S.Shaw	negative	-36.5137	175.397675	Dead
StephanieShaw	640	Leiopelma archeyi	unknown	StonyBayMoehau	Waikato	New Zealand	2007	TqPCR	2	0	S.Shaw	S.Shaw	negative	-36.528495	175.418424	Dead
StephanieShaw	641	Leiopelma archeyi	male	StonyBayMoehau	Waikato	New Zealand	2007	TqPCR	1	0	S.Shaw	S.Shaw	negative	-36.528481	175.418256	Dead

StephanieShaw	642	Leiopelma archeyi	unknown	StonyBayMoehau	Waikato	New Zealand	2006	TqPCR	1	0	S.Shaw	S.Shaw	negative	-36.529948	175.408069	Dead
StephanieShaw	643	Leiopelma hochstetteri	male	StonyBayMoehau	Waikato	New Zealand	2008	TqPCR	1	0	S.Shaw	S.Shaw	negative	-37.125884	175.207548	Dead
StephanieShaw	644	Leiopelma hochstetteri	unknown	StonyBayMoehau	Waikato	New Zealand	2008	TqPCR	2	0	S.Shaw	S.Shaw	negative	-37.125884	175.207548	Dead
StephanieShaw	645	Leiopelma pakeka	female	MaudIsland	Marlborough	New Zealand	2008	histology	8	0	J.Germano	S.Shaw	negative	-41.028543	173.888898	No
StephanieShaw	646	Litoria aurea	female	RangitotoStation	Waikato	New Zealand	2009	histology	1	1	L.Daglish	S.Shaw	positive	-38.339429	175.438054	Dead
StephanieShaw	647	Litoria raniformis	female	TeKuiti	Waikato	New Zealand	2008	TqPCR	1	1	L.Daglish	S.Shaw	positive	-38.30625	175.15325	Dead
StephanieShaw	648	Litoria aurea	male	Waitakere	Auckland	New Zealand	2008	histology	1	0	unknown	S.Shaw	negative	-36.850424	174.54254	Dead
StephanieShaw	649	Leiopelma pakeka	male	MaudIsland	Marlborough	New Zealand	2008	histology	8	0	J.Germano	S.Shaw	negative	-41.028543	173.888898	No
StephanieShaw	650	Litoria raniformis	female	UpperMoutere	Nelson	New Zealand	2009	isolation	2	2	M.Stratton	S.Shaw	positive	-41.243744	173.0453592	Sick
StephanieShaw	651	Litoria raniformis	male	UpperMoutere	Nelson	New Zealand	2009	isolation	1	1	M.Stratton	S.Shaw	positive	-41.243744	173.0453592	Sick
StephanieShaw	652	Litoria aurea	female	Silverdale	Waikato	New Zealand	2010	TqPCR	5	5	V.Carruthers	S.Shaw	positive	-37.802392	175.338621	Sick
StephanieShaw	653	Litoria aurea	male	Silverdale	Waikato	New Zealand	2010	TqPCR	1	1	V.Carruthers	S.Shaw	positive	-37.802392	175.338621	Sick
StephanieShaw	654	Litoria raniformis	female	RangitotoStation	Waikato	New Zealand	2010	histology	3	3	L.Daglish	S.Shaw	positive	-38.339429	175.438054	Dead
StephanieShaw	655	Litoria aurea	female	BlockhouseBay	Auckland	New Zealand	2008	TqPCR	1	0	B.Westera	S.Shaw	negative	-36.92336	174.7	Dead
StephanieShaw	656	Litoria raniformis	unknown	RangitotoStation	Waikato	New Zealand	2008	TqPCR	1	1	L.Daglish	S.Shaw	positive	-38.339429	175.438054	Dead
StephanieShaw	657	Leiopelma archeyi	female	Moehau	Waikato	New Zealand	2002	histology	1	1	S.Carver	S.Shaw	positive	-36.528778	175.389218	Dead
StephanieShaw	658	Leiopelma archeyi	male	TapuBell	Waikato	New Zealand	2002	histology	1	1	S.Carver	S.Shaw	positive	-36.9867	175.5814	Dead
StephanieShaw	659	Litoria aurea	unknown	GodleyHeadsChristchurch	Canterbury	New Zealand	1999	histology	5	1	B.Waldman	S.Shaw	positive	-43.591084	172.798719	Dead
StephanieShaw	660	Litoria aurea	female	unknown	Northland	New Zealand	2001	histology	1	1	R.Gill	S.Shaw	positive			Dead
StephanieShaw	661	Litoria spp	unknown	unknown	Northland	New Zealand	2002	histology	1	1	R.Gill	S.Shaw	positive			Dead
StephanieShaw	662	Litoria raniformis	unknown	Kaitaia	Northland	New Zealand	2002	histology	1	1	P.Anderson	S.Shaw	positive	-35.11733	173.267559	Dead
StephanieShaw	663	Litoria spp	unknown	Oakura	Northland	New Zealand	2002	histology	1	1	P.Anderson	S.Shaw	positive	-35.39114	174.343983	Dead
StephanieShaw	664	Litoria aurea	unknown	WhakanekenekeStream	Waikato	New Zealand	2002	histology	1	1	R.Chappell	S.Shaw	positive	-36.742333	175.500741	Dead
StephanieShaw	665	Leiopelma archeyi	male	TapuBell	Waikato	New Zealand	2002	histology	1	1	S.Carver	S.Shaw	positive	-36.9867	175.5814	Dead
StephanieShaw	666	Litoria aurea	male	HamiltonZoo	Waikato	New Zealand	2006	histology	1	1	J.Wallace	S.Shaw	positive	-37.77415	175.21717	Dead
StephanieShaw	667	Litoria ewingii	unknown	PurakanuiKaikourai Valley	Otago	New Zealand	2008	isolation	6	6	P.Bishop	S.Shaw	positive	-45.777741	170.612615	Sick

StephanieShaw	668	Leiopelma hochstetteri	unknown	KomataReefs	Waikato	New Zealand	2008	TqPCR	1	0	A.Haigh	S.Shaw	negative	-37.354938	175.739642	No
StephanieShaw	669	Leiopelma hochstetteri	unknown	ManganukuStream	Bay of Plenty	New Zealand	2008	TqPCR	2	0	A.Haigh	S.Shaw	negative	-38.28358	177.387156	No
StephanieShaw	670	Leiopelma hochstetteri	unknown	ManganukuStream	Bay of Plenty	New Zealand	2008	TqPCR	1	0	A.Haigh	S.Shaw	negative	-38.282357	177.387309	No
StephanieShaw	671	Leiopelma hochstetteri	unknown	ManganukuStream	Bay of Plenty	New Zealand	2008	TqPCR	1	0	A.Haigh	S.Shaw	negative	-38.282165	177.387377	No
StephanieShaw	672	Leiopelma hochstetteri	unknown	ManganukuStream	Bay of Plenty	New Zealand	2008	TqPCR	1	0	A.Haigh	S.Shaw	negative	-38.281607	177.387103	No
StephanieShaw	673	Leiopelma hochstetteri	unknown	ManganukuStream	Bay of Plenty	New Zealand	2008	TqPCR	2	0	A.Haigh	S.Shaw	negative	-38.28831	177.390115	No
StephanieShaw	674	Leiopelma hochstetteri	unknown	ManganukuStream	Bay of Plenty	New Zealand	2008	TqPCR	4	0	A.Haigh	S.Shaw	negative	-38.288361	177.390428	No
StephanieShaw	675	Leiopelma hochstetteri	unknown	ManganukuStream	Bay of Plenty	New Zealand	2008	TqPCR	3	0	A.Haigh	S.Shaw	negative	-38.288421	177.390511	No
StephanieShaw	676	Leiopelma hochstetteri	unknown	ManganukuStream	Bay of Plenty	New Zealand	2008	TqPCR	1	0	A.Haigh	S.Shaw	negative	-38.288186	177.390531	no
StephanieShaw	677	Leiopelma hochstetteri	unknown	TarawaereStrmKauranga Valley	Waikato	New Zealand	2008	TqPCR	2	0	A.Haigh	S.Shaw	negative	-37.076571	175.672266	No
StephanieShaw	678	Leiopelma archeyi	unknown	OngohiHutTeMoehau	Waikato	New Zealand	2001	histology	2	1	R.Norman	S.Shaw	positive	-36.53856	175.390738	Dead
StephanieShaw	679	Leiopelma hochstetteri	male	HunuaRanges	Auckland	New Zealand	2002	TqPCR	1	0	S.Shaw	S.Shaw	negative	-37.044984	175.197134	Dead
StephanieShaw	680	Leiopelma hochstetteri	male	HunuaRanges	Auckland	New Zealand	2003	TqPCR	1	0	S.Shaw	S.Shaw	negative	-37.044984	175.197134	Dead
StephanieShaw	681	Leiopelma hochstetteri	unknown	Maungatautari	Waikato	New Zealand	2004	TqPCR	1	0	S.Shaw	S.Shaw	negative	-38.035834	175.568796	Dead
StephanieShaw	682	Leiopelma hochstetteri	male	HunuaKMA	Auckland	New Zealand	2005	TqPCR	1	0	S.Shaw	S.Shaw	negative	-37.044711	175.203996	Dead
StephanieShaw	683	Leiopelma hochstetteri	male	HunuaKMA	Auckland	New Zealand	2008	TqPCR	1	0	S.Shaw	S.Shaw	negative	-37.044711	175.203996	Dead
StephanieShaw	684	Leiopelma hochstetteri	unknown	HunuaKMA	Auckland	New Zealand	2008	TqPCR	1	0	S.Shaw	S.Shaw	negative	-37.044711	175.203996	Dead
StephanieShaw	685	Leiopelma hochstetteri	unknown	HunuaKMA	Auckland	New Zealand	2008	TqPCR	1	0	S.Shaw	S.Shaw	negative	-37.044711	175.203996	Dead
StephanieShaw	686	Leiopelma archeyi	unknown	TapuBell	Waikato	New Zealand	2010	TqPCR	9	0	B.Bell	S.Shaw	negative	-36.986667	175.581389	No
StephanieShaw	687	Leiopelma hochstetteri	unknown	TapuBell	Waikato	New Zealand	2007	TqPCR	5	0	B.Bell	S.Shaw	negative	-36.986667	175.581389	No
StephanieShaw	688	Leiopelma hochstetteri	unknown	TapuBell	Waikato	New Zealand	2008	TqPCR	1	0	B.Bell	S.Shaw	negative	-36.986667	175.581389	No
StephanieShaw	689	Leiopelma hochstetteri	unknown	TapuBell	Waikato	New Zealand	2010	TqPCR	1	0	B.Bell	S.Shaw	negative	-36.986667	175.581389	No

StephanieShaw	690	Leiopelma archeyi	unknown	Whareorino	Waikato	New Zealand	2007	TqPCR	19	5	L.Daglish	S.Shaw	positive	-38.406335	174.799466	No
StephanieShaw	691	Leiopelma archeyi	unknown	Whareorino	Waikato	New Zealand	2007	TqPCR	11	1	L.Daglish	S.Shaw	positive	-38.406335	174.799466	No
StephanieShaw	692	Leiopelma hochstetteri	unknown	Waiau	Waikato	New Zealand	2008	TqPCR	1	0	A.Haigh	S.Shaw	negative	-36.807351	175.541398	No
StephanieShaw	693	Leiopelma archeyi	unknown	Whareorino	Waikato	New Zealand	2010	TqPCR	15	1	L.Daglish	S.Shaw	positive	-38.406335	174.799466	No
StephanieShaw	694	Leiopelma archeyi	unknown	Whareorino	Waikato	New Zealand	2010	TqPCR	23	0	L.Daglish	S.Shaw	negative	-38.406539	174.798509	No
StephanieShaw	695	Leiopelma archeyi	unknown	Whareorino	Waikato	New Zealand	2010	TqPCR	32	2	L.Daglish	S.Shaw	positive	-38.380199	174.794017	No
StephanieShaw	696	Leiopelma archeyi	unknown	Whareorino	Waikato	New Zealand	2010	TqPCR	41	3	L.Daglish	S.Shaw	positive	-38.380097	174.793041	No
StephanieShaw	697	Leiopelma archeyi	unknown	Whareorino	Waikato	New Zealand	2008	TqPCR	9	0	L.Daglish	S.Shaw	negative	-38.4065	174.7985	No
StephanieShaw	698	Leiopelma archeyi	unknown	Whareorino	Waikato	New Zealand	2008	TqPCR	7	0	L.Daglish	S.Shaw	positive	-38.3802	174.7940	No
StephanieShaw	699	Leiopelma archeyi	unknown	Whareorino	Waikato	New Zealand	2008	TqPCR	5	1	L.Daglish	S.Shaw	positive	-38.3801	174.7930	No
StephanieShaw	700	Leiopelma archeyi	unknown	Whareorino	Waikato	New Zealand	2009	TqPCR	1	0	L.Daglish	S.Shaw	negative	-38.4063	174.7995	No
StephanieShaw	701	Leiopelma archeyi	unknown	Whareorino	Waikato	New Zealand	2009	TqPCR	9	0	L.Daglish	S.Shaw	negative	-38.4065	174.7985	No
StephanieShaw	702	Leiopelma archeyi	unknown	Whareorino	Waikato	New Zealand	2009	TqPCR	49	10	L.Daglish	S.Shaw	positive	-38.380199	174.794017	No
StephanieShaw	703	Leiopelma archeyi	unknown	Whareorino	Waikato	New Zealand	2009	TqPCR	11	1	L.Daglish	S.Shaw	positive	-38.380199	174.794017	No
StephanieShaw	704	Leiopelma archeyi	unknown	Whareorino	Waikato	New Zealand	2009	TqPCR	34	3	L.Daglish	S.Shaw	positive	-38.3801	174.7930	No
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