Amphibian disease risks and the anthropogenic dispersal of invasive *Litoria* species.

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Thesis Abstract

The scope of this research is to provide a broad outline of the interaction between anthropogenic disease spread, risk of an invasive amphibian species establishing into the New Zealand environs, and prevalence of amphibian chytrid in wild introduced amphibian populations. The objective of this overview is to identify risk pathways that could threaten New Zealand's four endemic *Leiopelma* species of frogs.

Worldwide amphibian populations are in decline with an estimated 32.5% of amphibians globally threatened (IUCN 2008). New Zealand's four endemic amphibians *Leiopelma* spp. are high on the ICUN list of critically endangered animals, further, two of the three introduced tree frogs (*Litoria spp.*.) are listed endangered and vulnerable in their native home. Disease has been one factor implicated in the worldwide amphibian decline in particular the two diseases Chytridiomycosis and Ranavirus. Although Chytridiomycosis has had the most profound effect on the decline of amphibian species. The spread of such diseases is, at least in part, human-mediated through media such as the bait trade, food industry, and possibly the pet trade. To date, there have been no reports of Ranavirus in New Zealand amphibians. Conversely, amphibians chytrid fungus is widespread and has been implicated in the decline of the endemic *Leiopelma archeyi*. This makes amphibian chytrid an ideal disease to model disease transmission with particular reference to the anthropogenic movement of amphibians.

The two main goals of this Ph.D. were to investigate specific anthropogenic mediated risks of spreading disease using the pathogen *Batrachochytrium dendrobatidis* (Bd), which is responsible for amphibian chytrid fungus, as a modality to model this. Included in this will be the enquiry into how Bd entered New Zealand and how it spread so quickly via the movement of *Litoria spp.*. Furthermore, to look at invasive amphibian species incursion risks by evaluating previous border seizures.

Currently, it is unknown how the amphibian chytrid entered New Zealand and whether New Zealand's borders are a high-risk entry pathway for amphibian disease. Examining the anthropogenic dispersal of *Litoria* in New Zealand and developing systems that reduce the risk of introducing disease into naive populations is an important role in ensuring the long-term survival of New Zealand's endangered *Leiopelma* spp. frogs. The presence of Bd in New Zealand has been recorded but the prevalence of the pathogen in populations is unknown. Identifying the prevalence of infection within populations will provide insight into how populations of *Litoria spp.* are surviving Bd infection. Furthermore, this Ph.D. project will assess the risk of invasive exotic amphibians entering New Zealand and becoming naturalised.

Education is one of the important areas that will greatly help the plight of New Zealand's frogs. For education to be successful it needs to be targeted, therefore assessing risk areas of amphibian disease is imperative. Furthermore, understanding the public's knowledge of frogs in New Zealand will further help in the development of resource material and targeting the main groups where education is needed.

Key findings of this research are that the three species of *Litoria* frogs are moved around New Zealand in large numbers via the pet trade. The spread of amphibian chytrid has most likely been so rapid due to the frequency and volume of tadpoles and frogs being bought and sold. The pet trade thereby effectively and inadvertently is a major means of the unregulated translocation of *Litoria* amphibians throughout New Zealand. Results of this research also show there is a gap in the knowledge about amphibians in areas of husbandry, disease, species identification, and legal responsibilities in the ownership and containment of amphibians in New Zealand. Additionally, the introduction of a new disease is more likely to occur than the risk of an invasive species becoming established. Finally, the wild populations of *Litoria* frogs were surviving with a high prevalence of amphibian chytrid fungus in two of the three study sites in this research, the third site which had the presence of a reservoir species had low numbers of frogs present.

“Through dangers untold and hardships unnumbered, I have fought my way here to the castle beyond the Goblin City…”

Labyrinth 1986

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Dedication

This thesis is dedicated to my Supervisor Professor Phil Bishop, aka amphibian-Phil, Phil the frog man. Not only an amazing knowledgeable supervisor but an all-around great guy. I, like most of the amphibian world, will miss you. I am so sad you didn't get to see the completion of my thesis. Gone way too soon and way too fast. You will always be New Zealand's number one frog man.

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1 Introduction

Along with four species of critically endangered endemic frogs *(Leiopelma archeyi, Leiopelma pakeka, Leiopelma hamiltoni and Leiopelma hochstetteri*), New Zealand has three species of introduced frog (*Litoria spp*.). Unlike our endemic frogs, the introduced species (*Litoria aurea Litoria raniformis* and *Litoria ewingii*) have no conservation status or recovery plan in New Zealand, even though the IUCN (Red List) status for *L. raniformis* is endangered and *L. aurea* is vulnerable in Australia. All three introduced frogs are endemic to Australia, where two of the species, *L. aurea* and *L. raniformis* have seen dramatic declines in their native habitat. Although *L. aurea* was regarded as common in the 1960's, 30 years later it is listed as endangered in New South Wales (White & Pyke, 1996).

Amphibian declines primarily have an anthropogenic basis; habitat destruction, overexploitation, anthropogenic movement of frogs, and consequently the spread of disease, have all been implicated in the worldwide decline of amphibians (Alton & Franklin, 2017; Grant, Miller, & Muths, 2020). Global trade in wildlife poses the risk not only to the decline of wild populations and the spread of potentially invasive species, but also of spreading pathogens into naive environments and the development and spread of novel pathogens. Both *Batrachochytrium dendrobatidis* (Bd) and Ranavirus have been found in wild-caught tiger salamanders (*Ambystoma tigrinum*) used in the USA bait trade (Picco & Collins, 2008) and the North American bullfrog (*Rana catesbeiana*) sold in the international food trade (Schloegel et al., 2009). Although New Zealand has strict biosecurity regarding the importation of animals, including amphibians, and New Zealand's endemic frogs are protected under the NZ Wildlife Act 1953, the introduced frogs are not protected (Wildlife Act 1953 31 schedule 5) and their endangered status in their native home ranges are not recognised under New Zealand legislation. However, there is legislation under the NZ Conservation Act 1987 65 26ZM1 that states, "No person shall transfer live aquatic life or release live aquatic life into any freshwater": although this is rarely enforced with regard *Litoria* spp. Lack of guidelines, clear regulations, or the enforcement of these to prevent the release of *Litoria* spp.. could pose a risk to the endemic *Leiopelma* spp.. Indeed, the introduction of Bd into New Zealand has been implicated in the sudden and significant reduction (88.0% decline) of *L. archeyi* in Coromandel between 1996-2001 ((Ben D. Bell, Carver, Mitchell, & Pledger, 2004). It has been speculated that commercial collectors of *Litoria* may have inadvertently introduced the amphibian chytrid fungus into a wild population of *Litoria* in Christchurch that was confirmed positive for Bd in 1999 (P Bishop, 2000; Waldman et al., 2001). This population was the first recorded incidence of the disease in New Zealand. Previously it had been noticed populations of *Litoria* spp. had declined although no firm conclusion of why these population declines occurred was reached (PJ Bishop, 1999).

Litoria spp.

Litoria is a genus of tree frogs belonging to the Hylidae family of frogs. Hylidae are categorised into three sub families of which *Litoria* belong to the Pelodryadinae group the Austro-Papuan group of tree frogs (http://animaldiversity.ummz.umich.edu/site/accounts/classification/path/Pelodryadinae.html). Typically, tree frogs (Hylidae) are at least partially arboreal during their life cycle, but some are terrestrial or semi aquatic. *Litoria* are found throughout Australasia and vary greatly in appearance and habitat. Characteristics of the genus are horizontal pupils and lack of pigmentation on their eyelids. There are approximately 65 species of *Litoria* in Australia with the rest being found throughout Wallacea. They lay their eggs in or near water where their larval stage lives before metamorphosing into adult frogs (Ehmann, Tyler, & Australian, 1996; Tyler & Knight, 2011)

Litoria Species Distribution in New Zealand

New Zealand's 3 species of *Litoria*, (*L. aurea*, *L. raniformis* and *L. ewingii*) have all been introduced from Australia where they are endemic. The three *Litoria* species were intentionally and repeatedly released in New Zealand during the 1860's with the aim of establishing wild populations. Sometimes several attempts were made to establish these populations (Phillip Bishop, 2008). Both *L. raniformis* and *L. ewingii* are found throughout the North and South Islands. The distribution of *L. aurea* appears to be restricted to the top half of the north Island although there are populations further south and iNaturalist reports sightings of *L. aurea* in the South Island. The habitat requirements for *L. ewingii* are wide: they can inhabit bush, pastureland and urban areas (Shaw et al., 2013). They tolerate cold and dry habitats and have been recorded above the treeline in the South Island (Gill & Whitaker, 1996). They have even been observed in dry tussock land at Macraes Flat Dunedin (Chris Wedding *pers comm.*) and can survive freezing (Rexer-Huber, Bishop, & Wharton, 2015). Meanwhile, both *L. aurea*, *L. raniformis* (Bell frogs) are morphologically and ecologically similar with both preferring farmland, swamps lakes and other areas with slow moving water either near bush or in settled areas (Gill & Whitaker, 1996).

The least widespread of the introduced *Litoria* spp., *L. aurea* are a highly dispersive species and have been recorded to travel over 10km in the breeding season (G. Pyke & White, 2001) In New Zealand,

they are restricted to suitable habitats above 380 latitude (Bishop 2008) as conditions are possibly to cold further south for them except in small ecological niches in the lower North Island, although the website iNaturalist NZ has reported sightings of individuals in the South Island these are rare and in some instances confused with *L. raniformis*. Any human mediated attempts to colonise *L. aurea* populations further south are likely to fail although this could change with global warming.
Introduced species can become invasive and out-compete native species (A. Cunningham, Daszak, & Rodriguez, 2017). To date there is little evidence that any of the endemic *Leiopelma* species are being threatened by the widespread *Litoria* species. There is a single report of L. archeyi hind legs being in the stomach contents of a *L. aurea* in Whareorino forest population. There is also sightings of *L.aurea* in open areas of Whareorino forest alongside *L. archeyi* (Thurley & Bell, 1994). However, reports of the two species living sympatrically are not common in the literature. This is probably due to the different habitat requirements of the two genera. *Litoria* are aggressive generalist predators and adult frogs will readily cannibalise smaller individuals and cannot be kept in captivity in groups with variable sizes as the large individuals will eat smaller individuals (pers. obs.). As *Leiopelma* are smaller than *Litoria* it is expected that they would be eaten if the two came into contact if there was a size difference. *Litoria* spp. also share habitats with other New Zealand endemic species: insects, lizards, and geckos all which could be predated upon by *Litoria* spp.

Litoria spp. have been affected by another introduced species, the mosquito fish, *Gambusia holbrooki*. These fish were introduced into New Zealand in the 1930's and since then have become widespread throughout the North Island and the top of the South Island (Ling 2004). Australian populations of bell frogs have seen population declines due to predation by this species (Clemann & Gillespie, 2013; Graham Pyke & White, 2000). It has been proposed that *Gambusia* are also having a negative effect of New Zealand populations (G. Pyke, 2002; White & Pyke, 1996). Pyke et al. (1996) found *L. aurea* were more likely to be found at new sites that were considered highly disturbed environments. They state it is not clear why frog breeding is enhanced in these disturbed habitats. It is postulated that predator species like *Gambusia* had not yet established in these sites giving the frog larvae better survival rates.

Pathogen Pollution

Population declines encompass many different features; the five major reasons postulated are habitat reduction, overexploitation, chemical pollution, introduction of pest species and disease. Each of these may solely be responsible for declines or the decline may be multifactorial. Generally, introduced species are considered to be a risk to endemic species due to predation or competition. However, the co-introduction of pathogens also poses a considerable risk to wildlife (A. A. Cunningham, Daszak, & Rodriguez, 2003). It has been speculated that the fungal pathogen *Bd* was inadvertently dispersed around the world when in the 1930s the resistant species *Xenopus laevis* started to be distributed worldwide to laboratories and zoo's (Fisher & Garner, 2020; Symonds, 2005; Weldon C, Du Preez L.H, Hyatt A.D, Muller R, & Speare R, 2004).

Pathogen pollution described by Cunningham et al. (2003) (Daszak, Cunningham, & Hyatt, 2000; McKenzie & Peterson, 2012) is the introduction of pathogens, specifically driven by anthropogenic activities into new populations and new environs. Pathogen pollution may involve horizontal disease transmission between similar species or as in the case of amphibian chytrid fungus may involve a whole class of amphibia. Cunningham et al. (2003) note the pathogen does not always rely on transportation into new areas. They use the common brush tail possum (*Trichosurus vulpecular*) as an example of hosting a pathogen that crossed ecological barriers. The possums provide a new host for bovine tuberculosis (Tb) and aid the spread of bovine Tb into other hosts such as farmed deer *Cervus elaphus* (red deer). The wild possum therefore provides a bridge for the transmission of Tb between two farmed animals helping the disease spread more readily.

Amphibian chytrid is a pathogen that has bridged evolutionary boundaries such as geographic isolation through the anthropogenic movement of amphibians. The movement of infected animals into new environments has resulted in amphibian chytrid spreading across the globe and being regarded as a leading factor in global amphibian declines (Skerratt et al., 2007). Currently, it appears the anthropogenic movement of amphibians out of Asia via trade is disseminating amphibian chytrid globally (Fisher & Garner, 2020).

Chytridiomycosis

Chytridiomycosis is caused by the amphibian chytrid fungus *Batrachochytrium dendrobatidis* (Bd) and develops solely in keratinised cells where it causes hyperplasia and hyperkeratosis. The *Bd* zoospores require water or moist conditions to survive and can survive for eight weeks or more outside the hosts body (IUCN 2005). Bd will not survive desiccation (Johnson M. L. & Speare R., 2003). At least part of its lifecycle (zoospore stage) is aquatic, during this stage the zoospores are motile, meaning one mode of disease transmission is through infected water. In moist environments, Bd zoospores are resilient and can survive and remain infective in water for up to seven weeks (Johnson M. L. & Speare R., 2003). The reproductive zoosporangia live in the keratinised layers of amphibian skin eventually causing mortality in susceptible species through osmotic imbalance resulting in cardiac arrest (Voyles et al., 2007).

Unlike other chytrids which are saprobic, Bd is a parasitic chytrid that has prolonged viability in moist conditions. Furthermore, there are examples of amphibians acting as asymptomatic carriers and therefore vectors for disease, these include Rana *catesbeiana* (Hanselmann et al., 2004; Mazzoni et al., 2003; Schloegel et al., 2009). *Xenopus spp*.. (Weldon C et al., 2004) and *Ambystoma tigrinum* (Collins J.P, Brunner J. L, Jancovich J. K, & Schock D. M, 2004). These species all appear to exist with sub-clinical infections. Axolotl (*Ambystoma mexicanum*) are another amphibian that is an asymptomatic carrier (Frías-Alvarez et al., 2008). Axolotls are commonly sold throughout New Zealand in pet stores where infected individuals could be a source of contamination to other amphibians if strict hygiene protocols are not followed. The three species from the order Urodela present in New Zealand are: Japanese fire belly newt (*Cynops pyrrhogaster*), Chinese fire belly newt (*Cynops orientalis*) and the European alpine newt (*Ichthyosaura alpestris*) are all considered potential disease reservoir hosts (B. D. Bell, 2016; Kolby et al., 2014; Smith et al., 2018). It is unclear the extent to which other aquatic animals are asymptomatic carriers and/or disease reservoirs for amphibian chytrid, although crayfish (L. A. Brannelly, McMahon, Hinton, Lenger, & Richards-Zawacki, 2015; Paulraj et al., 2016), freshwater prawns (Paulraj et al., 2016), freshwater shrimp (Rowley, Alford, & Skerratt, 2006), and birds (Burrowes & De la Riva, 2017; Garmyn et al., 2012) have all been implicated in the maintenance and transmission of Bd in and between habitats (Prahl, Wilson, Giles, & Craddock, 2020). Host species that are asymptomatic can maintain diseases within a population by acting as a reservoir (L. Brannelly et al., 2018). The 2013 discovery of the European Alpine newt *I. alpestris* in the North Island of New Zealand could be a possible reservoir for Bd. The newts are known to be asymptomatic when infected with Bd (Arntzen, King, Denoel, Martinez-Solano, & Wallis, 2016)

Tadpoles can also provide a mode of transmission for amphibian chytrid to be spread into new habitats as they are easily collected and moved via anthropogenic means. As tadpole's skin does not contain keratinised cells and they don't appear to be as affected by Bd in their pre-metamorphic state. Tadpoles carry Bd in their mouthparts as this is the only keratinised tissues in their pre-metamorphic bodies (Daszak et al., 1999)). "*Bd infection frequently caused substantial mortality in post-metamorphs, and mostly sub-lethal, but not-insignificant, effects in tadpoles.*" (Kilpatrick, Briggs, & Daszak, 2010), p. 113). This would make tadpoles ideal dispersers of Bd as they are readily transported around the country and may show no clinical signs of Bd infection. Translocation of apparently healthy tadpoles by members of the public and by the pet trade has spread Bd to new localities in New Zealand

(Waldman et al., 2001). Recording the anthropogenic dispersal of *Litoria* throughout New Zealand will help assess disease risks and assist in disease management.

Schloegel et al. (2009) noted there was a seasonal variation in the prevalence of amphibian chytrid in the USA pet trade, with higher prevalence being recorded in cooler months. They suggest further research into this factor to determine the reason for these fluctuations. Determining if there is any seasonal variation in the presence of amphibian chytrid in the pet trade will be influential in developing best practise protocols for this industry.

Initial observations of current hygiene practises in the pet trade suggested there were a widespread substandard hygiene protocols and a lack of general knowledge about New Zealand's frogs i.e. what species are available and correct identification of species. These factors along with what I observed as an almost universal lack of knowledge about not releasing frogs and tadpoles into waterways are indicative of the need to better educate amphibian owners and provide traders with advocacy material for them to distribute. They are also indicative of the potential risks this industry poses to New Zealand's native fauna by releasing an invasive introduced species.

"*The impact of chytridiomycosis on frogs is the most spectacular loss of vertebrate biodiversity due to disease in recorded history*" (Skerratt et al., 2007), pp.125). It has been estimated that chytridiomycosis has caused the decline or extinction of 200 species of frog from 14 families and two orders since 1980 (Kriger & Hero, 2009; Skerratt et al., 2007). It spreads rapidly and infects a wide range of host species (Daszak, Cunningham, & Hyatt, 2003) which makes it a major threat to amphibians worldwide. In Australia it is reported to spread 100km per year (Alexander & Eischeid, 2001) and has been held responsible for major amphibian declines in otherwise pristine habitats (Skerratt et al., 2007).

Presently, the international trade of amphibians in and out of New Zealand is highly restricted and extremely limited. Since the introduction of The Biosecurity Act in 1993 neither *Ambystoma mexicanum* (axolotl) nor *Xenopus* have been imported into the country (Viki Melville *pers. comm*. 2010). Conversely, the national trade in amphibians is highly active and completely unregulated. Live amphibians are regularly posted via the mail service or couriered throughout the country in large numbers, one breeder reportedly selling over 2000 tadpoles in one season (*Pers Obs*. December 2011). The trade largely involves the introduced *Litoria* species, axolotl and newts with *Litoria spp.* being the most frequently sold through Trade Me^{TM} a New Zealand-based online selling website (*Pers. Obs*. December 2011).

The presence of Bd in New Zealand has been known since 1999 (Waldman et al., 2001). There have been four strains isolated that all appear to relate to the BdGPL line. Vigilance at the border needs to be maintained, as like many pathogens Bd has many strains that are continually evolving with varying pathogenicity

Objectives

The overall aim of this study is to evaluate current biosecurity risks from potential disease pathways using amphibian chytrid as a model system and the risk of introducing another invasive amphibian species. The use of Bd as a model is relevant for several reasons, firstly the pathogen Bd has cause the most widespread decline in amphibians globally, it has been stated to have caused one of the most catastrophic losses of biodiversity to be recorded (Scheele et al., 2019). The pathogen Bd will provided an excellent model to look at disease spread for several reasons. Primarily, as it affects a wide range of hosts, whose vulnerability varies widely from being asymptomatic carriers/reservoir hosts to it being lethal (Skerratt et al., 2007). Furthermore, life stage can affect susceptibility or resistance to the pathogen (Bakar A., et al., 2016; Johnson M. L. & Speare R., 2003; Sauer et al., 2020). Amphibians are easily transported around and are transported in large numbers for pets, food and bait (Wombwell et al., 2016). Bd zoospores can live for extended periods outside the host in moist conditions, so can easily be spread via contaminated equipment or via the substrate or water which infected animals are transported in (Johnson M. L. & Speare R., 2003).

Assessing the risk of the introduction of an exotic invasive amphibian species will provide further insight to the threats that face our native frog species. To achieve this the specific objectives of this research are divided into four main sections. The focus of each of the four sections of this thesis are:

Section 1: Biosecurity: Assessing disease and invasion risk in amphibians in New Zealand.

New Zealand is renowned for its biosecurity effort, yet although substantial resources are directed to keep out exotic species and pathogens there are still breaches of border security. In relation to amphibians there have been both pathogen Bd and exotic amphibians that have managed to evade border controls. The aim of this section of my thesis is to identify risk areas for incursions of alien amphibians and the pathogen Bd. The amphibian chytrid fungus being the focal disease for this research as it has breached border security and has resulted in a decline of both *Litoria spp*. and possibly *Leiopelma archeyi*.

Objective 1.1 Identify the risk of the historic introduction of amphibian chytrid into New Zealand via the borders.
Objective 1.2 Identify risk areas at the border for the introduction of alien amphibians.
Objective 1.3 Identify high risk amphibian species entering New Zealand regarding Bd infection and risk of becoming naturalised.
Objective 1.4 Identify any seasonal patterns of introductions of amphibians at the border.

Section 2: Online sales of *Litoria spp*. in New Zealand.

Frogs and tadpoles are popular pets in New Zealand. The extent to which they are bought, kept, and moved around is unknown and unregulated. This section aims to evaluate the numbers and movements of *Litoria* species via an online New Zealand based selling site, Trade Me, and the risks of spreading the pathogen Bd.

Objective 2.1 Quantify the extent of the online amphibian trade in New Zealand using Trade Me as an indicator.
Objective 2.2 Identify *Litoria* species sold online New Zealand and the availability and location of each species.
Objective 2.3 Investigate risk areas in the online trading of *Litoria spp.*

Section 3: Pet shop sales, husbandry practises and knowledge of amphibians.

Pet shops frequently sell a variety of amphibian species. With regards to disease spread they provide a bottleneck as many species from many sources come into close contact with each other. To mitigate any spread of disease a high standard of hygiene is imperative. Pet shops also have the scope to provide customers with quality information regarding husbandry and animal ownership responsibilities. However, to provide such information they would have to have the pertinent knowledge themselves. The aim of this section is to assess these factors so risk areas can be identified and mitigated.

Objective 3.1 Assess the general knowledge of people who are involved in the amphibian pet trade.
Objective 3.2 Assess the husbandry practises in the pet trade.

Objective 3.3 Identify which amphibian species are sold through pet shops and the number of individuals sold.

Section 4: Identify the presence, transmission, and maintenance of Bd in New Zealand amphibians.

The first record of Bd in New Zealand was in 1999. Subsequently, it has been recorded in all three of the exotic *Litoria spp*. and both populations of *Leiopelma archeyi* (Whareorino and Coromandel). The other three species of *Leiopelma*; *L. hochstetteri, L. pakeka*, and *L. hamiltoni* have not had any recorded Bd infections. The risk to *Leiopelma* frogs appears minimal however the introduction of a new more virulent strain could change this. Therefore, it is pertinent to look at transmission risks, the obvious one being through the pet trade. Furthermore, identifying where Bd is found in wild populations and understanding how it is being maintained in those populations will provide insight into the pathogen's effects on *Litoria* frog's survival in New Zealand.

Objective 4.1 Record the prevalence of Bd in *Litoria* in the pet trade.

Objective 4.2 Determine the prevalence of Bd in Zoo populations of amphibians.

Objective 4.3 Establish the prevalence of Bd in wild populations of *Litoria*.

Objective 4.4 Determine the prevalence of Bd in the containment species *Xenopus.*

Objective 4.4 Test the European alpine newt (an invasive alien species) of for the presence of Bd.

Objective 4.5 Assess the prevalence of Bd in *Litoria aurea* at two separate sites.

Objective 4.6 Compare a population of *Litoria aurea* with the presence of a reservoir species European alpine newt.

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2 Biosecurity: Assessing Disease and Risk of Invasion of Exotic Amphibians in New Zealand

Abstract

The Ministry of Primary Industries (MPI) is charged with keeping New Zealand safe from biological risk among other things such as food safety and sustainable use of resources. As an island nation, New Zealand's flora and fauna have a high rate of endemism due to species evolving in an isolated archipelago. As such this makes them vulnerable to both novel diseases and invasive species. The ICUN redlist lists the four Leiopelmatidae species of endemic frog as endangered. The risks range from *L. hochstetteri* being of least concern to *L. archeyi* being listed as critical, both *L. hamiltoni* and *L. pakeka* are considered vulnerable. Biosecurity efforts to mitigate invasion by alien species and disease is expensive, therefore defining high-risk areas and refining methodologies and best practices to have the best biosecurity outcomes in a cost-effective way is important.

The Ministry of Primary Industries keeps a record of all seized amphibians that have crossed New Zealand's borders. Along with each specimen that is preserved in 70% ethanol a detailed summary of information about the individual and its incursion details were recorded. This database provided a tool to assess the risk of invasion. Furthermore, as there were preserved specimens, the presence of disease could be evaluated. The disease amphibian chytrid fungus, *Batrachochytrium dendrobatidis* (Bd) was tested for in the seized amphibians for several reasons. Firstly, Bd is present in New Zealand however it is unclear how it arrived here. Secondly, Bd is easily spread via the movement of frogs, tadpoles, contaminated water, and equipment. Thirdly, funding would not allow for the testing of multiple diseases, and it was considered wide range testing of a single disease was more effective at looking at a disease transmission model rather than less thorough/comprehensive testing of multiple diseases especially since there is no evidence that they have breached New Zealand's borders.

The conclusions drawn from this research are the greatest risk to amphibians in New Zealand is from a novel disease. It would only take a single diseased individual to escape into the environment or be mishandled at the border for a disease outbreak to occur. The risk of an invasive species establishing accidentally is unlikely due to the low numbers of the same species entering, along with the reproductive and environmental requirements of amphibians. The exception to this would be deliberately smuggled species that were later released.

Introduction

The earth has moved into a new era, the Anthropocene, characterised by its sixth mass extinction event (Ceballos, Ehrlich, & Dirzo, 2017). This large-scale loss of biodiversity will have serious repercussions for humanity (Ceballos et al., 2017; Dullinger et al., 2013), impacting every aspect of human life (e.g., agriculture, health, and parasites, and pests). To date, current extinctions are largely a result of human activities, with key drivers being habitat destruction, overexploitation, global warming, and the introduction of exotic species. These drivers have caused local extirpations and global extinctions (May, 2010).

Between 1950 and 1999 there has been a fourteen-fold increase in global trade and with it an exponential increase in biological invasions (Nordström & Vaughan, 1999). Complicating the mitigation of this form of biological pollution is the conflict between global trade and the environmental autonomy of a nation (Hayes, 2003). Controlling and monitoring the movement of pest species and diseases associated with this global trade is an expensive process. In New Zealand, the costs are met through the commercial sector via levies on importers, exporters, and producers; they are also partially funded through the tax payer via the government (https://www.mpi.govt.nz/news-and-resources/media-releases/review-of-cost-of-food-safety-and-biosecurity-services/)

Risk assessment of bio-invasion and bio-pollution, and mitigating pest and disease control, are complex and involve a multitude of factors, many of which can be synergistic in their outcome(s). Factors such as volume and frequency of international trade and origin of goods, along with climatic and environmental factors, all impact risk. Risk factors along with the numerous invasive flora, fauna, and diseases that pose a threat to New Zealand's biodiversity, make the task of keeping New Zealand's unique and vulnerable flora and fauna and monetary valuable trade commodities safe, a complex and expensive operation. Historically, being an island nation has proven a dilemma for New Zealand: our highly specialised, endemic species having evolved over 60 million years of isolation yet are extremely susceptible to novel pests and diseases (Jay, Morad, & Bell, 2003). Three types of risks are associated with species incursions into a new environment: 1) establishment of an invasive species, 2) naturalisation of a benign species, and 3) transmission of diseases that impact existing native biodiversity. An invasive species is defined as a species that is: 1) non-native (or alien) to the ecosystem under consideration and 2) whose introduction causes or is likely to cause economic or environmental harm or harm to human health (Reaser et al., 2020).

Border biosecurity practices endeavour to protect New Zealand from the accidental or illegal importation of exotic fauna and flora. New Zealand has strict border controls that have been in place since the government implemented The Biosecurity Act in 1993 (http://www.legislation.govt.nz/act/public/1993/0095/164.0/DLM314627.html). Prior to 1993, the main focus of biosecurity in New Zealand was protecting primary producers from pests and disease and control primarily involved the use of quarantine (Whyte 2005). The Biosecurity Act aimed to provide a more comprehensive system of protection that encompassed the economy, environment, and human health. In New Zealand between 1992 and 2002, trade volumes increased by 76.0% and international passengers rose by 93.0% (Biosecurity strategy 2003 from Stats NZ 1991/2-2001/2). Currently, the Ministry for Primary Industries (MPI) oversees New Zealand's biosecurity. MPI was formed in 2012 and incorporates the previous Ministry of Agriculture and Forestry (MAF), the Ministry of Fisheries (MFish), and the New Zealand Food Safety Authority (NZFSA). The structure of New Zealand's biosecurity administration includes five separate yet integrated organisations responsible for border patrol, import health standards, quarantine, surveillance, and disease and pest emergency response (Hayden & Whyte, 2003), p. 271).

One of the most negatively impacted taxonomic groups are amphibians, where species loss has been rapid, intense, and worldwide (Skerratt et al., 2007). All of the major drivers mentioned above are applicable to the decline and extinctions of amphibian populations worldwide. However, the role of disease in the declines of natural populations is often overlooked (Preece et al., 2017). Despite, diseases having an ever-increasing role in population declines (ibid.). Of particular concern for amphibians is amphibian chytrid *Batrachochytrium dendrobatidis* (Bd), which appears to have quickly spread and become increasingly virulent due to anthropogenic assisted movement of amphibians (Farrer et al., 2011). Hence, Bd can provide a model for discovering how a pathogen can evolve and adapt in response to human activities. Results from recent research propose that it is a pathogen that is exacerbated by the commodification and intentional and unintentional global transport of amphibians (Schloegel et al., 2012).

Chytridiomycosis is a disease caused by the fungus *Batrachochytrium dendrobatidis* (Bd). The chytrid fungi are an ancient family of saprophytic fungi that typically parasitise dead plant matter or other fungi. Bd has somehow jumped an evolutionary barrier and along with the more recently discovered *Batrachochytrium salamadrivorans* (Bsal) is the only known species of chytrid to parasitise vertebrates keratin (Fisher, Garner, & Walker, 2009). It has most likely evolved via the "mating between two non-identical but closely related, heterothallic, parental strains" (ibid. p.303). Bd is a diploid fungus with low genetic diversity and high heterozygosity suggesting a recent emergence (Fisher et al., 2009; James et al., 2009).

Although Bd has only ever been observed to reproduce asexually in laboratory situations, the recent discovery of four separate lineages including a hyper-virulent lineage suggests recombination does occur (Farrer 2011). The aetiology of amphibian chytridiomycosis involves the Bd zoospores embedding in the keratin of the amphibian epidermis causing disruption to osmoregulation and ultimately asystolic cardiac arrest and death (Voyles et al. 2009).

Since 1993, there have been several applications to import amphibians into New Zealand and between 2004 and 2015 there have been five permits issued and each permit covers multiple consignments over time (MPI *pers comm*.). All approved importations are for laboratory purposes, and none were approved for the pet trade or wild release of amphibians. This effectively minimises the introduction of amphibian disease into New Zealand via importation, as all approved permits require that animals be kept in containment facilities.

Chytridiomycosis provides a novel way to investigate disease transmission across vast distances within a globalised world. Attributes that make it a good candidate for transmission studies include the recent evolution of the disease and the occurrence of at least one hyper-virulent strain. This hyper-virulent strain in particular appears to be spread via anthropogenic movement of amphibians (Fisher et al., 2009). Furthermore, Bd has some asymptomatic host species, Bd can survive off the host for at least five weeks in a moist environment (Johnson M. L. & Speare R., 2003), and Bd varies in its morbidity across different life stages (Rosenblum, Voyles, Poorten, & Stajich, 2010). Furthermore, chytridiomycosis outbreak dynamics are linked with host skin bacterial community structure (Bates et al., 2018). Additional to these disease traits, infected amphibians are easily and widely transported globally via the pet trade, food trade, and smuggling.

It is frequently stated that Bd is found on every continent on earth (excluding Antarctica where amphibians do not exist). Olsen et al. noted it has been recorded in 56 0f 82 countries 68.0%. (Olson et al., 2013). The development of a community-led web database to record the location of the population where Bd is present provides a valuable tool for the understanding of the transmission of Bd on a global scale http://www.bd-maps.net/maps/ . Amphibian chytrid was first discovered in New Zealand six years after The Biosecurity Act (1993) came into effect. It was found in a population of *Litoria raniformis* inhabiting a pond near Christchurch in 1999 (Waldman et al., 2001). The original source of this first detection of the disease remains unknown, however, there has been speculation, albeit unsubstantiated, that it arrived and was spread via the pet trade (Waldman et al., 2001). Furthermore, there has been conjecture that the sudden decline observed in *Leiopelma archeyi* around 1996 was also due to the introduction of Bd into this population (Bell, Carver, Mitchell, &

Pledger, 2004). However, this has been questioned by (Bishop et al., 2009; Shaw et al., 2010), as there appears that *Leiopelma* have some resistance to Bd, laboratory experiments have demonstrated adult *Leiopelma archeyi* are able to survive Bd infections.

The origin and timing of the first introduction of chytridiomycosis into the New Zealand environs remains unknown. It is unlikely that it was introduced through legal importation of amphibians, as all recently permitted imports of amphibians are kept in containment facilities; furthermore, there were no legal amphibian importations between 1993 and the discovery of Bd in 1999 that were not containment facility applications. This leaves two possible scenarios, either it was present but undetected prior to The Biosecurity Act (1993), or it was introduced via a smuggled or accidental introduction of amphibians. Shaw et al. (2013) tested 152 museum specimens dating from 1930 to 1999 the time of the first record of Bd in New Zealand. Testing was performed on two *Leiopelma*: *L. archeyi* and *L. hochstetteri* as well as the 3 *Litoria spp*. currently, in New Zealand, all tested negative for Bd. This suggests Bd arrived via smuggled or accidentally introduced amphibians.

The Ministry of Primary Industries (MPI) is responsible for biosecurity in New Zealand and requires any overseas aircraft or sea craft to land at an approved destination or place of first arrival (POFA). There are 14 approved POFA for aircraft consisting of seven international airports, military airports, and regional airports, however only Auckland, Wellington, and Christchurch international airports are approved for POFA of live animals and commercial cargo. There are 22 seaports with POFA approval; these include recreational, cargo, military, and passenger ports. Each sseaporthas specific requirements for approved vessels and types of cargo, baggage, and passengers (specific details are available at [https://www.mpi.govt.nz/news-and-resources/resources/registers-and-lists/places-of-first-arrival-seaports/).](https://www.mpi.govt.nz/news-and-resources/resources/registers-and-lists/places-of-first-arrival-seaports/)

The 2003 Biosecurity Strategy, on page 23, highlighted the absence of “a more proactive approach … in assessing emerging threats, to enable identification of potential pests and pathways and implementation of measures to prevent their entry, spread, and establishment” (Hellström, Moore, & Young, 2003). This research has attempted to address that gap for New Zealand amphibians. We used the MPI collection of seized amphibians from border control, to provide insights into the incursion pathways of amphibians and assess the risk of an exotic amphibian naturalising and potentially becoming invasive. Further, we assessed the risk of disease transmission and directly tested for the presence of the panzootic fungus Bd.

This study aims to evaluate current biosecurity risks from invasive amphibian species and potential disease pathways using amphibian chytrid as a model system. New Zealand’s border security procedures monitor all vessels transporting cargo, baggage, and passengers from overseas ports to New Zealand. Data used to meet this aim come from the MPI collection of herpetofauna intercepted as part of standard border biosecurity practice over a 14-year period (2000 to 2013). This database contains the most complete and robust records of herpetofauna entries into New Zealand and will be used to assess disease risk associated with growing global transport and inform future biosecurity practice in New Zealand and worldwide. The disease of focus is chytridiomycosis caused by the pathogen Bd because it is in New Zealand, and it is unclear how it arrived. Furthermore, Bd has had the most profound and devastating effect globally of any disease (Scheele et al., 2019)

Methods

Database, Background and Structure

Since August 2000, the late Tony Whitaker collected, recorded, and stored herpetofauna that were intercepted at New Zealand's borders or post-border. The collection includes all Amphibia intercepted until his death in 2014. Both accidental and illegal incursions were included in the collection. The collection includes the individual specimens along with relevant information documented at the time. The information was recorded in two forms: primarily in a Microsoft Excel spreadsheet (version 9.0, 2000) and a secondary paper record within the container of each ethanol-stored specimen. The two types of information were not equally detailed; the data recorded on the excel spreadsheet was more comprehensive and more consistent; therefore, these data were used in this study. There were also existing data on 43 individuals from 39 incursions collected prior to Whitaker managing the collection in August 2000. These pre-August 2000 data were less comprehensive and are mostly included in Gill et al. (2001).

The Whitaker (MPI) data contains 30 separate variables, and only a small number were incomplete for all specimens and these specimens were excluded from the current analysis where the data was not complete. The variables included in this analysis were considered important in contributing to risk of invasion and disease risk and were as follows: 1) Species name using the *binomial nomenclature* including, *Family, Genus, and Species*. This variable was used to identify the species more likely to cross borders and also to determine whether species that were entering were asymptomatic hosts or a species affected by Chytridiomycosis. 2) *Country of origin*: Information as to the origin of the specimen provides information on which country the amphibians came from; this can be used to determine the likelihood of Bd exposure. It also identifies the numbers from specific countries. 3) *Type* of introduction (accidental or smuggled) can provide information to identify risk pathways. 4) *Number of specimens* found per incursion was included to assess risk of any species becoming naturalised. 5-7) *Entry town, Entry point, Location found*, were included to determine movement once specimens arrived. *Entry point* indicated what type of port i.e., air or sea the specimen/s enter via. 8) *Cargo and Cargo system* were included to assess by what means incursions were entering. 9) The *Border/Post-border* variable identified where the individuals were intercepted and was used as a measure of the effectiveness of border patrol. 10) *Date* of the incursion (month/year) was used to analyse seasonality of incursions. 11) *Life stage* of the specimen (alive/dead) provided insights into risk of naturalisation into the New Zealand environment.

Seasonality of country of origin for incursions was examined. It is important for two reasons: amphibians are more likely to travel during wetter, more humid months and during breeding and post-breeding periods (Seebacher & Alford, 1999; Vasconcelos & Calhoun, 2004). Bd infection is also associated with these times and shows seasonality both in occurrence (Kinney, Heemeyer, Pessier, & Lannoo, 2011), infection load (Kriger & Hero, 2007), and virulence (Berger, Marantelli, Skerratt, & Speare, 2005; Murray, Skerratt, Speare, & McCallum, 2009). Overall, there is a higher chance of susceptible individuals being infected and having a higher infection load in cooler, moister seasons (Murray et al., 2011; Murray et al., 2009). Seasonality was determined by categorising the data as follows: summer = December-February autumn = March-May, winter= June-August and spring = September-November.

Of the thirty variables within the dataset, fifteen categories of risk factor where defined: Species, Origin, Smuggled/ Accidental, Number, Entry Town, Distance Travelled, Location Found, Air /Sea, Cargo, Border/ Post Border, Alive/ Dead. All of these categories differ in the potential risk they pose in facilitating a) species invasion and/or b) disease transmission. There are some clearly high-risk categories like number of individuals (both per interception and overall) and interception of live animals that increase the risk of both invasion of species and disease. In contrast, risk of disease incursion is high regardless of life stage. Separate categories may have a multiplicative effect on risk when combined. Two examples illustrate this: first the origin of a species influences the ability of an animal to survive both the journey and its new habitat, and second, the risk of being released into a new environment is increased by not being discovered at the border and the distance it was transported once crossing the borders.

To assess the potential risk of the amphibian establishment a comparison was made between post border and border intercepts using the three main types of entry under the variable cargo system Container, Airfreight and, Baggage. The container was specifically related to goods being brought into the country via seaports, whereas airfreight was via airports. Baggage was specifically related to personal items bought in by tourists, immigrants or travellers returning home.

Bd Infection Identification

All amphibian specimens held and collected by MPI from 2001 until 29th August 2013 were tested for *Batrachochytrium dendrobatidis* (Bd) using polymerase chain reaction (PCR). Specimens were couriered in five batches from the MPI collection held by Whitaker to Massey University (Auckland Campus) and a total of 201 specimens were received. Specimens were stored in individual glass containers of 70.0% ethanol; each container had an identification code that corresponded to information in the database provided. Additional information about the sample was written on a waterproof paper tag and stored within each container. Upon arrival at Massey, each sample was checked and processed. Containers typically held a single specimen, but a few held up to 8 specimens. Each individual was photographed dorsally and ventrally with the container identification number for later identification if required. In containers with multiple specimens, the individuals were labeled with the container catalogue number and a unique alphabetic identifier.

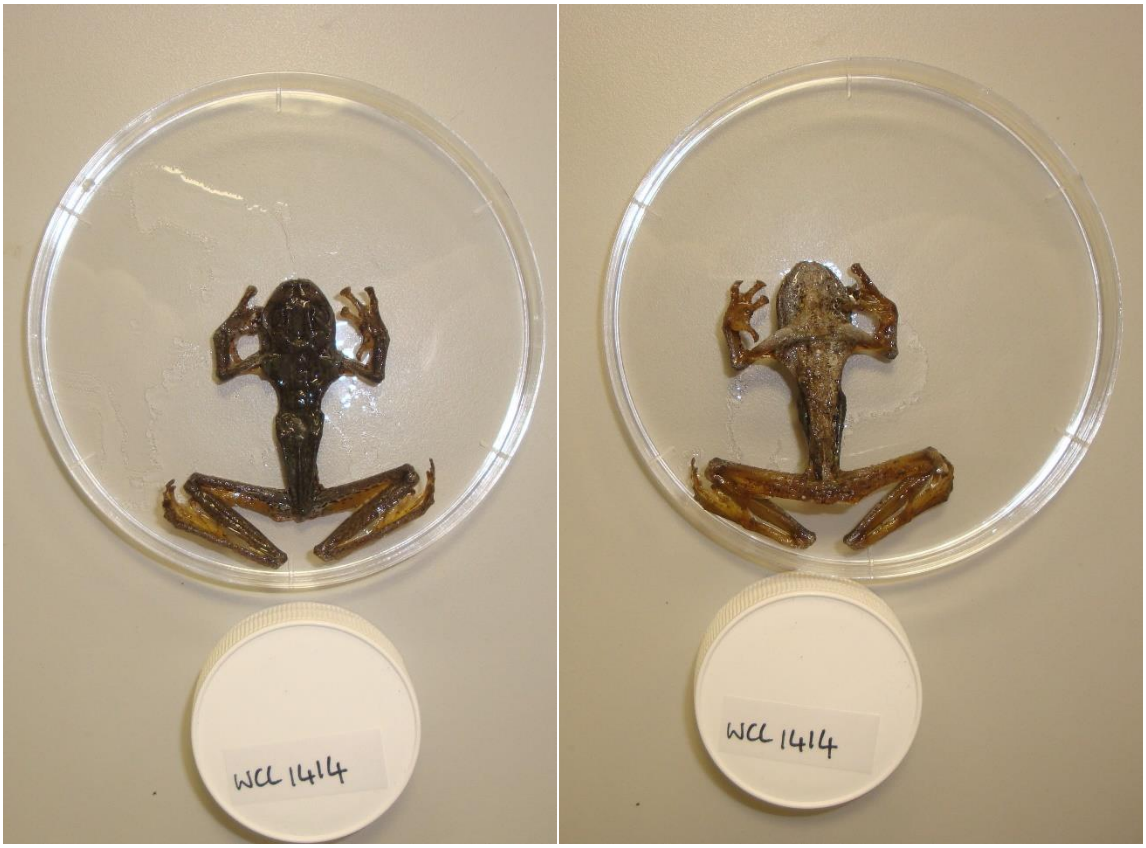

Figure 2.1 Example of frog specimen. MPI specimens were photographed with ID number allocated by MPI. This individual was desiccated prior to preservation

To minimise cross-contamination, each specimen was processed separately: first photographed then swabbed with two types of swabs. The swabbing technique used follows Hyatt et al. (2007); in summary, the swab was rotated and wiped four times along each side of the animal from beneath the forelimb to the top of the hind limb; four times medially and laterally on each thigh and finally four times on the plantar side of each hand and foot. The first swab was done with a standard MW100 swab and the second used a flocked swab. The MW100 is the standard swab used when swabbing for chytridiomycosis on live or recently deceased amphibians. As the sample contained specimens that had been stored for up to 12 years in ethanol, and some specimens were in poor condition, a flocked swab for seven random individuals was also used. Flocked swabs have a higher absorbency and was more abrasive, therefore likely to lift more cells from the sample (Soto-Azat et al., 2009).

The labelled swabs were then frozen for subsequent DNA extraction. After swabbing, a 3mm x 3mm tissue sample was taken from the right lower ventral side of the pelvis of each individual for later histology work. Two specimens were too small to take a skin sample and a number of the smaller specimens had tissue samples less than 3mm x 3mm removed. All tissue samples were labelled and stored in 5ml Eppendorf tubes containing 70.0% ethanol until histological examination was completed.

Swabbed Samples and PCR Summary

The DNA was extracted using Prepman^{TM}. Each swab was placed in a sterile Eppendorf ^{TM} and 50ul of Prepman^{TM} was added. The sample was vortexed for 30 seconds and spun down at 8000 rpm for 30 seconds then placed in a thermocycler for ten minutes at 100^{o}C. After cooling the sample was centrifuged for one minute at 13000rpm and aliquoted into a new sterile Eppendorf then frozen at -20^{o}C. All the MW100 swabs had the DNA extracted, additionally, there were seven samples that were rerun because results were inconclusive. For these seven the corresponding flock swabs were used and had DNA extracted followed by RT-PCR to amplify the genetic material.

Histology

For 33 specimens where skin samples were taken, a histologic examination was performed. These samples wherwereedded in the substrate at Massey University and sent to James Cook University in Queensland, Australia, to be cut dyed and slides prepared. The samples that had previously been stored in 70.0% ethanol, were embedded in paraffin and sectioned at 5 µm, and stained with haematoxylin and eosin, the stratum corneum was scanned at x200 or x400 power. Ten samples had to be re-embedded at James Cook University and then subsequently cut, dyed, and analysed.

Data Extraction and Statistical Analysis

Descriptive summaries of the frequency of incursions for each category of each variable are presented. The frequency distributions of all incursions in relation to specific combinations of the 12 variables from the database were compared using Chi-squared tests of independence. A significance level of 0.05 was used for all tests. The Chi-squared test was obtained from https://www.socscistatistics.com/tests/chisquare2/default2.aspx .

Effect sizes were described using histograms and summary tables of values. European newts were excluded from many of the analyses as more than 3000 were captured from an eradication attempt of an established incursion population within New Zealand many years after the incursion occurred. The 13 included in the database were therefore not an indicator of the number of animals within the original incursion.

To look at seasonal patterns, first monthly frequencies of interceptions for the ten-year period from 2003-2012 were used with totals for each month pooled across these years. Months were then combined into seasons (summer = December to February; autumn = March to May; winter = June to August; Spring = September to November).

To compare incursion distances, three variables were considered for this analysis: entry town, entry point and location found. Only incursions that had information on these three variables (and were found after point of entry) were included in this analysis. For the 'location found' variable, the central point of the stated location listed on google maps was used as the point of location coordinates as the exact address was not available.

Results

The Origins of the Incursions

The dataset contained 191 instances where the country of origin was recorded for 236 incursions. There were 30 different countries of origin. Of these, 16 countries were the source for two or more incursions, the remaining 14 countries were the origin for a single incursion event. Australia (n=74) was the most common country of origin with over three times more incursion events than the next most common origin, Fiji (n=19), see Figure 2.2.

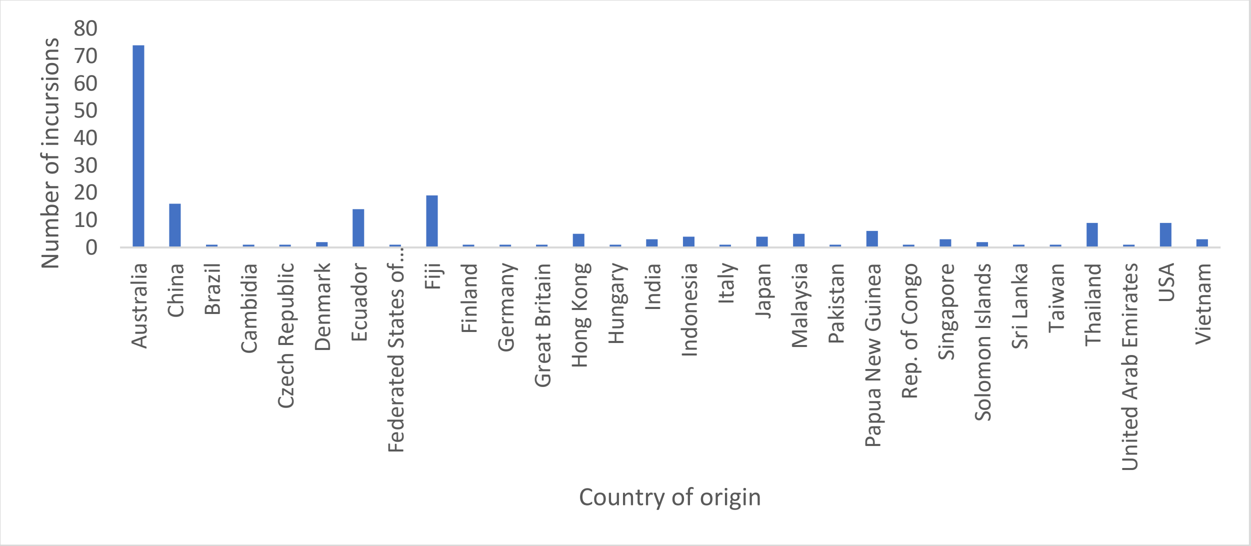

Figure 2.2 Number of incursions and country of origin

Species Intercepted

Family, genus, and species of the interceptions were recorded when specimens were identifiable. Of 236 incursions, 226 were identified to family, 221 were identified to genus, and 177 to species. Only twenty-five percent (59/236) were not identified to species level. Of the nine different families, the most common were Hylidae n=102 followed by Bufonidae n=86. The most frequent genus represented in the data was *Bufo* n=78, followed by *Litoria* n=68, *Kaloula* n=12, and *Hyla* n=10. The species most frequently occurring were *Rhinella marina* n=39, followed by *Duttaphrynus melanostictus* n=29, *Litoria caerulea* n=20, *Litoria gracilenta* n=10, see figure 2.3.

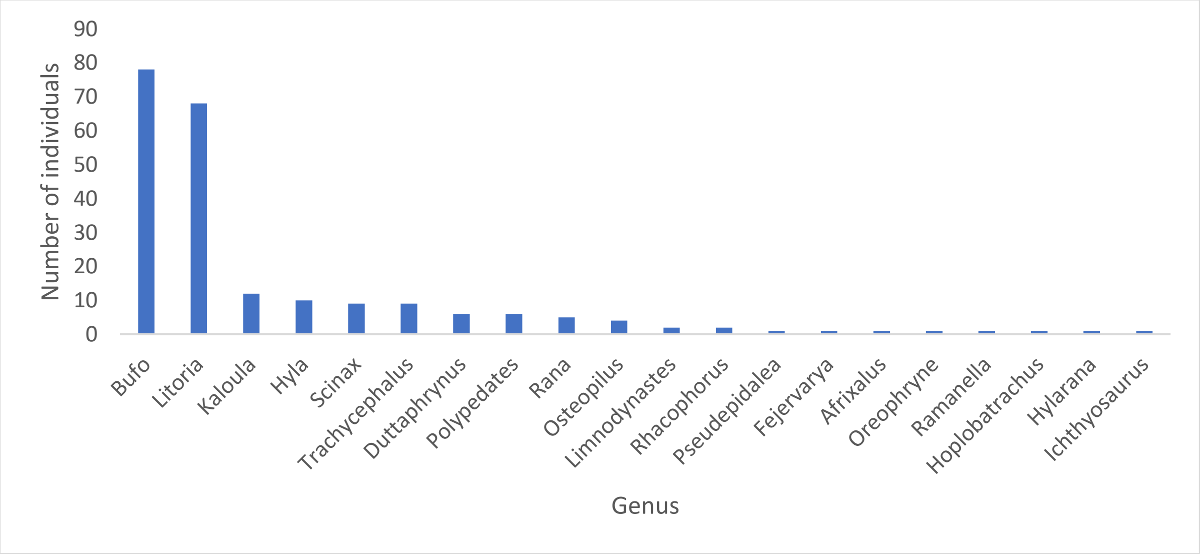

Figure 2.3 Number of each genus intercepted.

Risk of Bd for Each Incursion

For 27 of the 30 countries of origin, at least one amphibian population in that specific country had tested positive for *Batrachochytrium dendrobatidis (*Bd), the pathogen causing chytridiomycosis. The only countries without confirmed Bd infection were the Solomon Islands and the Federated States of Micronesia (where climatic conditions are favourable for Bd), and The United Arab Emirates (UAE), where the dry, hot climate is generally unsuitable for Bd to survive in the wild. However, the two species intercepted originating from the UAE were both from the Bufonidae; a family that has been found positive for Bd.

A total of 45 unique species are present in the dataset. Of these species, 31 have been previously reported as species being susceptible to Bd infection. For the remaining 14 species there was no reported data found to confirm Bd infections, however Bd infection has been identified in all but three of the genera (*Oreophryne, Pseudepidalea*, and *Scinax*). Hence, the majority of amphibians entering New Zealand have the potential to be infected with Bd.

Number of Individuals Per Incursion

The total number of individuals intercepted, and data recorded for the period between March 1978 and August 2013 was 317 (this includes only 13 of the more than 3,000 European newts *Ichthyosaurus alpestris* that were part of a major seizure of released and naturalised individuals: only 13 were kept in the collection).

Excluding the European newts of which over 3000 have been found in the wild. The number of individuals per incursion ranged widely (1 to 54) with larger numbers (6 to 54) associated with smuggling events. All accidently introduced individuals were in small cohorts (≤4). The majority (87.6%) of incursions were single individuals, followed by n=2 (8%), n=3 (2.2%), n=4 (0. 9%), and n>4 (1.3%). Smuggled individuals were rare occurrences (1.3%) however larger numbers of individuals were involved and all individuals were alive.

Life State

A high proportion of the amphibians intercepted were live animals: 72.4% of individuals (excluding the newts). Despite this, no new amphibian populations (excluding the newts) have established as postulated by Gill et al. (2001). This is probably due to New Zealand's unfavourable climatic conditions in relation to the species that have arrived. Nonetheless, although it appears that the risk of a new species of amphibian establishing in New Zealand is low, it is of concern that there are an increasing number of amphibians being intercepted at the border: the increase of 0.7 incursions a year between 1978 and 2012 (figure 2.4).

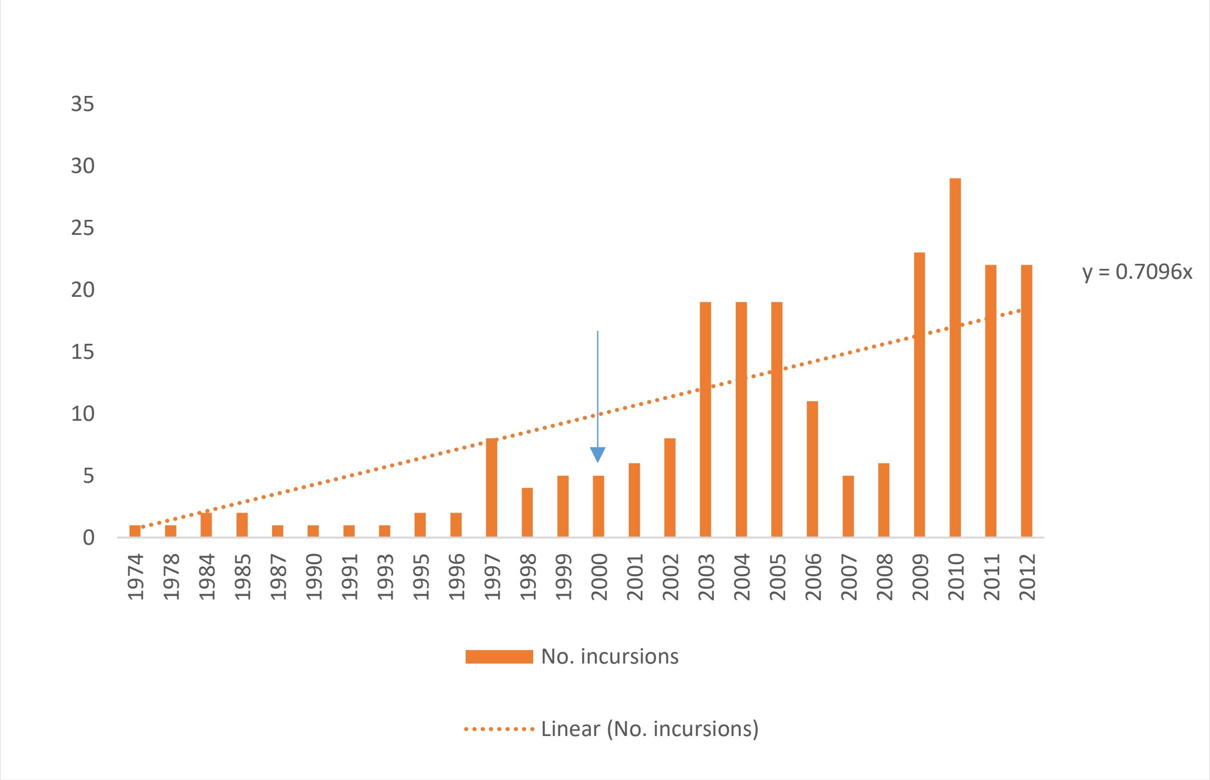

Figure 2.4 Number of incursions per year 1974-2012. The blue arrow signifies the year the data and collection became more consistent: when T. Whitaker started keeping it.

Of the 317 individuals intercepted in 130 separate seizures, 226 were captured live, 81 deceased, and 10 individuals did not have life stage recorded. The 13 European newts are not included in the graph figure 2.5 as the collection only holds a small portion of the actual numbers seized. The number of incursions mostly reflected the number of individuals with the exception of smuggled individuals. Figure 2.5 compares the frequencies of live/dead incursions to the number of live/dead individuals. There were three instances of smuggled individuals being seized in 2009: these where all interconnected. The graph only includes complete years and only those entries which stated whether the incursions/individuals were recorded as alive or dead.

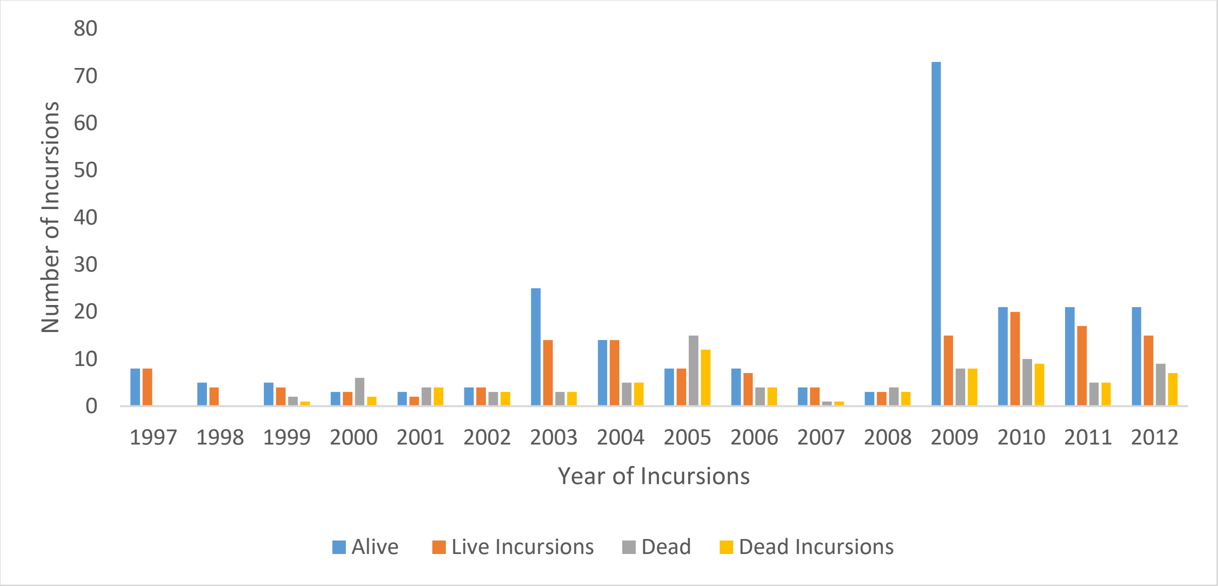

Figure 2.5 Annual numbers of individuals and incursions divided into life stage at time of interception.

To assess the risk of naturalisation of a species of amphibian the data were examined by species with more than one incursion per calendar year. There were nine species within this category (Figure 2.6), *Rhinella marina, Scinax quinquefasciata, L. caerulea, Duttaphrynus melanostictus, Kaloula pulchra, L. gracilenta, Hyla gratiosa, Osteopilus septentrionalis,* and *L. peronei. Rhinella marina* is notable for having multiple years of multiple individuals being intercepted by Ministry of Primary Industries (MPI).

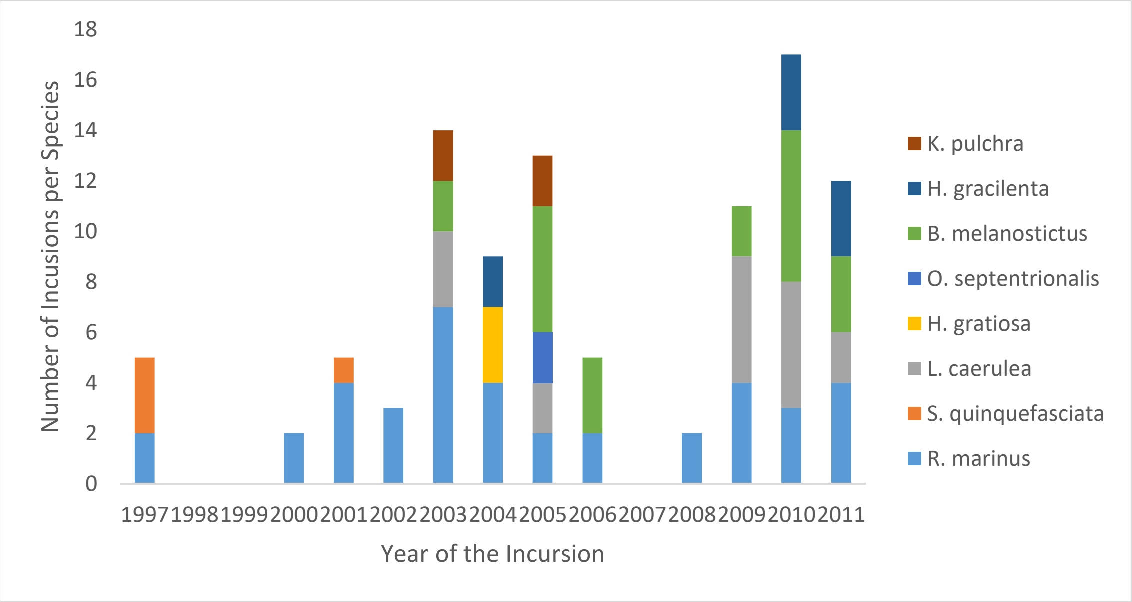

Figure 2..6 Frequency of incursions of species that were intercepted more than once per calendar year.

Evidence of Seasonality

Looking at the data between 2000 and 2012 there was no evidence of seasonality in the number of incursions. Due to the incursions originating from both northern and southern hemispheres there would be a likelihood of any seasonality being counteracted by grouping the northern and southern hemispheres as their seasons are opposite. There was, however, monthly variability of incursions being intercepted in New Zealand see Figure 2.5.

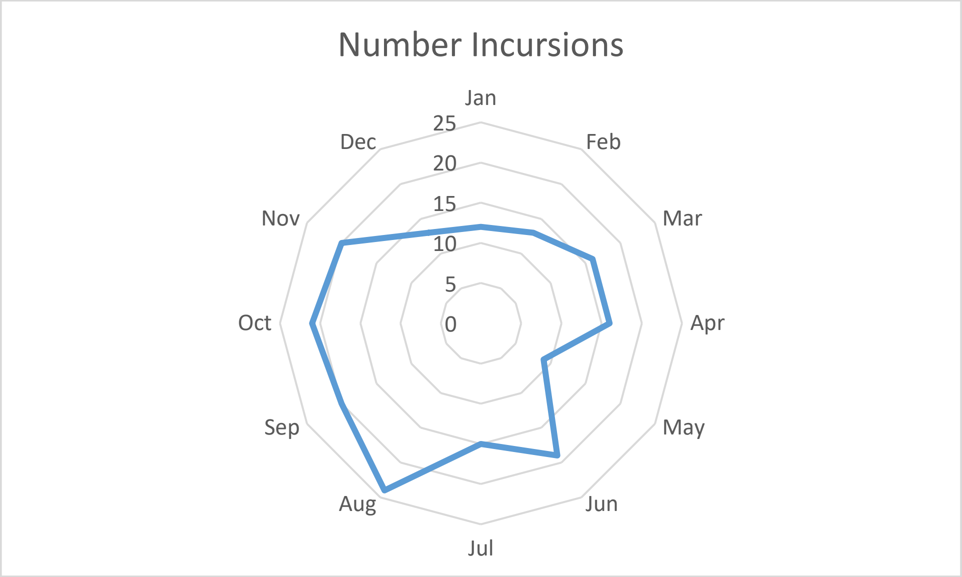

Figure 2.7 Incursions by month.

When the northern and southern hemispheres were analysed separately seasonality was apparent in incursions. The interceptions of amphibians from the northern hemisphere were significantly more likely during spring months, while southern hemisphere incursions were higher in autumn months X^2(3, N=199) =9.2993 p= 0.026.

The seasonality of the intercepts from the country of origin was explored to see if there was a higher risk of amphibians leaving their country of origin in a particular season. To do this northern hemisphere seasons were swapped accordingly: winter was equivalent to the southern summer and spring was equivalent to the southern autumn and vice versa. The countries that spanned both hemispheres were excluded and the appropriate season at origin was assigned, time for transit was also included, I assumed a duration of one month for the voyage between origin and destination for northern hemisphere individuals based on the average voyage time from the northern hemisphere of 21 days. For example, an individual that was intercepted in New Zealand in November would be presumed to be in transit from October in the northern hemisphere. Re-analysing the data using a one-month lag-time and season corrected demonstrated a significant effect due to seasonality;

northern hemisphere amphibians were significantly more likely to leave their country of origin in summer and autumn, furthermore they were less likely to leave in winter. The southern hemisphere data only showed significantly more incursions in spring X^2 (3, N=199) =9.28, p=0.03. Comparing the whole data set including the countries that span both hemispheres (equatorial) the effect of season was not significant at 0.05.

The data of alive/dead incursions in relation to the New Zealand seasons showed significant seasonality in the life stage, with individuals more likely to be found dead in spring (September-November), and summer (December – February). The season with the most marked difference is winter with many more live incursions and fewer dead incursions. χ^2 (3, N=223) =10.48, *p*=0.02). A reason for this could be better body condition as they reach winter metabolism slows down and energy needs are less so better body condition is maintained. Furthermore, the weather is cooler and damper, so desiccation is less likely.

Mode of Entry and Location of Seizures

The most common way for amphibians to enter the country was via seaports n=124 (67.8 %) compared with airports n=59 (32.2%). This likely reflected the volume of goods and type of goods associated with each type of entry. Greater tonnage entered the country via seaports compared with airports during the decade 2000- 2010 (Table 2.1).

The dataset included records of where the animals were seized: post border or border. There were 83 post border seizures and 102 border seizures. Actual numbers of individuals seized were higher post-border n=151 compared to at the border n=115. More individuals were intercepted alive (n=118) than dead (n=33) post border, although this was not statistically significant (χ^2 1= 3.58, P>0.05).

When the location of interception (post border or border) for the two modes of entry (air and sea) were compared there was no significant difference i.e., the ratio of incursions being detected post-border and border were the same for both modes of entry: seaports post border n=49, border n=75; airports post border n=29, border n=30, χ^2 (3, N=183) = 1.518, p=0.22.

1	2 000/01	3 001/02	4 002/03	5 003/04	6 004/05	7 005/06	8 006/07	9 007/08	10 008/09	11 009/10	12 010/20 11
13 To tal Airports (Tonnes)	14 8046	15 2654	16 8204	17 5734	18 06089	19 06104	20 04446	21 04583	22 8540	23 0891	24 6676
25 To tal Seaports 26 (T onnes)	27 407364 3	28 536379 5	29 604295 0	30 761032 3	31 905750 5	32 801328 2	33 839456 0	34 921905 1	35 734890 2	36 724718 5	37 845732 0
38 To tal Sea and Air (Tonnes)	39 416168 9	40 544644 9	41 613115 4	42 770605 7	43 916359 4	44 811938 6	45 849900 6	46 932363 4	47 743744 2	48 733807 6	49 855399 6
50 no of Incursions	51	52	53 2	54 8	55 2	56 6	57	58	59 3	60 6	61 2
62 no of individuals	63	64	65 4	66 8	67 2	68 9	69	70	71 3	72 6	73 4
74 ^{a} (http://www.transport.govt.nz/ourwork/tmif/freighttransportindustry/ft010/)											

Table 2.1 Yearly volume of imported goods in tonnes compared to the number of individuals intercepted and the number of incursions.

Incursion Distances

The subset of data where incursion distances were known consisted of 102 separate instances where one or more individuals were intercepted at a location beyond point of entry. Incursions were often seized a considerable distance from their point of entry. I assumed that the greater the distance detected from the point of entry, the higher the risk of disease transmission into the New Zealand environs. Distances travelled, via human transport, once entering the country ranged from 2.89 km to 1028.69 km; the average distance travelled from point of entry to interception was 135 km. The majority of movements were within one of the two main islands of New Zealand n=95, while only a few movements were between islands n=6. All inter-island movements were from north to south.

Risk assessment

When comparing the three different cargo systems and whether the incursion was post border or at the border, amphibians were significantly more likely to be found post border in baggage than in containers or in airfreight. Conversely, amphibians were much less likely to be found at the border in baggage χ^2 (2, N=154) =9.05 p=0.01. Interestingly, amphibian incursions in baggage post border would have primarily been surrendered voluntarily.

To determine whether amphibians were more frequently discovered in food/produce items the cargo was divided into 2 types: Food/live plants, non-food and looked at incursions post border and border. Incursions were significantly more likely to be found post border in food/live plant imports χ^2 (1, N=121) =8.66 p=0.003.

Disease Analysis: Presence of Bd

There were 201 individuals tested for Bd infection. With the exception of the European newts *Ichthyosaurus alpestris*, (Laycock, Whitaker, Bishop, Skerratt, & Brunton, 2015) there was an absence of Bd positive specimens from all of the PCR samples. Of the initial 13 *Ichthyosaurus alpestris* tested five tested clear positive for Bd, while a further two were equivocal with 2/3 replicates showing positive.

A selection of 33 individuals considered most likely to be infected with Bd were then tested for evidence of Bd sporangia within skin using histology. The samples were all *Litoria* species *(L. caerulea* n= 31 *L. rubella* n= 1 and *L. fallax* n=1). *L. caerulea* was chosen as it is a species that is particularly susceptible to Bd infection (Berger et al., 2005). In addition, the individuals used originated from areas of Australia where Bd is present (Murray et al. 2011). The *L. rubella* and *L. fallax* samples were included in the testing as they were found in the same incursion and therefore stored together with the *L. caerulea* specimens. Twenty-nine of the specimens had been found alive and had been subsequently euthanised, hence they appeared to be in good condition for histology. The specimens were all collected between March 2003 and August 2004 so were all within a period when chytridiomycosis was already present in New Zealand.

The samples had been stored in 70.0% ethanol for up to 13 years. Samples were placed into 70.0% ethanol either after euthanasia or upon being sent to MPI for storage, there may have been some time lag between being found and being given to MPI for subsequent storage, resulting in poor preservation for histological analysis. None of the samples had been fixed in formalin prior to storage. Although to the naked eye the samples looked good, the immediate storage in ethanol meant the skin was brittle and dehydrated and not in the best condition for histological analysis (Speare et al., 2005)

Of the 31 skin samples tested, eighteen samples were of sufficient quality for testing. Unfortunately, the condition of the skin samples varied, with many being of poor quality and missing the epidermis or autolysed. There was no conclusive evidence of Bd infection via histological testing.

Discussion

My incursion dataset showed both low propagule numbers (average n=17.6 live incursions per year) and low propagule size (< 12.0% of incursions consisted of two or more individuals). This suggests accidental introductions of amphibian species that have been intercepted by MPI would be a low risk of becoming either established or invasive in New Zealand. Initially the data suggests, at least for amphibians coming into New Zealand, propagule number would have more of a risk on whether naturalisation and/or an invasion event would happen. This is based on the data showing repeated small numbers of a particular species being intercepted (propagule number) as opposed to incursions that are of a large number of a single species (propagule size). The MPI data were explored further by looking at numbers of incursions and number of each species within each incursion between 1998 and 2011. Several species: *R. marina, L. caerulea* and *D. melanostictus*, do indeed pose a risk of accidently becoming established in New Zealand as there is regularly more than one incursion each year for each of these three species. Furthermore, there has been an overall increase in incursions and multiple incursions of some species. Interestingly, there is no correlation between the volume of trade and the number of amphibian incursions; this suggests amphibians are getting through the country-of-origin screening more often. A possible reason more incursions are being missed when trade volumes are larger is because the number of staff and resources available for screening are not increased comparatively.

The species that is the biggest threat of naturalisation is the cane toad *R. marina* as it is the most frequently found incursion. This species has become a pest in several countries including Australia (Lever, 2001; Phillips, Brown, Webb, & Shine, 2006; Sutherst, Floyd, & Maywald, 1995) where the majority of our amphibian incursions originate. The cane toad can survive in the northern parts of New Zealand and is a know carrier of Bd (Brannelly, Martin, Llewelyn, Skerratt, & Berger, 2018). Therefore, *R. marina* poses both the risk of becoming invasive and introducing different strains of Bd.

The second most regularly intercepted species at New Zealand's borders is *L. caerulea*. *Litoria caerulea*, the green tree frog, is a popular pet species (Latheef et al., 2020) and was one of the species in this data set that was smuggled into New Zealand. *Litoria caerulea* is likely to be able to survive in the northern, warmer areas of New Zealand because it has a wide range of habitat tolerances (Buttemer, 1990)and its native habitat overlaps with *L. aurea*, a species now naturalised in the North Island. As a popular pet species, *L. caerulea* and, if naturalised, it would likely be targetted by the pet trade and quickly spread further if released into the wild. With a life history similar to the two other *Litoria* spp. currently present in New Zealand, it is likey to become established as a pest in the warmer regions. Additionally, *L. caerulea* would provide another opportunity to carry, transmit and maintain amphibian chytid as it is known to be susceptible to Bd.

Duttaphrynus melanostictus (Asian Common Toad) is the third most regularly intercepted amphibian by New Zealand's border biosecurity. Although New Zealand's current climate would be marginal for naturalisation of this species to it is known to tolerate to a broad range of habitats and is currently increasing in numbers worldwide (http://www.iucnredlist.org/details/54707/0). With trends in global warming and this species adaptability, northern New Zealand could provide suitable future habitat as this species is highly adaptable (Moore, Francois Solofo Niaina Fidy, & Edmonds, 2015). If it became established, it would pose a considerable threat to the New Zealand environment and also human health due to its toxicity (Invasive Species Council, 2014).

(Simberloff, 2009) noted that “Increasing propagule size enhances establishment probability primarily by lessening effects of demographic stochasticity, whereas propagule number acts primarily by diminishing impacts of environmental stochasticity” (p 81). The species with incursions of the highest propagule size were all smuggled species except for one group of 11 individuals of *L. caerulea*. If, as (Simberloff, 2009) states, an increase in propagule size correlates to decrease in the effects of demographic stochasticity, smuggled amphibians would pose the greatest risk to become invasive if the species smuggled were released into a suitable environment. Although smuggled individuals may have a low propagule number, the effects of this on environmental stochasticity would be nullified as smuggled species would not have the environmental pressures of a wild released cohort and if they were ultimately released they could be released in higher numbers, and into a favourable environment. The fact that they are relatively rare events (only three events were recorded in the 35 years of the data set 1978- 2013) and of those three instances there was one successful release event that was naturalised, the population of European newts discovered in 2013, points to smuggled species being the highest risk area for invasive amphibian species establishment.

A high proportion of the amphibians intercepted were live animals: 72.4% of individuals (excluding the newts). Despite this, no new amphibian populations (excluding the newts) have been established as postulated by (Gill, Bejakovtch, & Whitaker, 2001). This is probably due to New Zealand’s unfavourable climatic conditions in relation to the species that have arrived. Nonetheless, although it appears that the risk of a new species of amphibian establishing in New Zealand is low, it is of concern that there are an increasing number of amphibians being intercepted at the border, particularly since 2000. This could be a result of either more comprehensive screening and/or increases in the anthropogenic movement of amphibians.

Competition from an invasive amphibian would appear to be low risk to New Zealand endemic anurans. New Zealand’s four endemic species of frog are unique in their habitat requirement as three of the four species (*Leiopelma archeyi*, *L. hamiltoni* and *L. pakeka*) prefer cool (<18^{0}C) moist terrestrial habitats (Cree, 1989; Newman, Crook, & Imboden, 1978; Shaw et al., 2010). Two of the four endemic species (*L. hamiltoni* and *L. pakeka*) are only found on offshore islands. *L. hochstetteri* is semi-aquatic and prefers temperatures between 15.3 - 20.9 ˚C (Easton, 2015) would be at most risk as it is the most aquatic of the four Leiopelma spp.. Although the risk of establishment of invasive amphibian into New Zealand appears to be a low risk, disease risk and disease pathways of incursions are an important issue.

The introduction of novel amphibian diseases poses a serious risk to the four endemic *Leiopelma spp*. of frog. All four *Leiopelma spp.* are endangered, three species are confined to four main populations Hamilton's Island (*L. hamiltoni*), Maud Island (*L. pakeka*), Whareorino Forest and Coromandel (*L. archeyi*). *L. hochstetteri* is found in fragmented and isolated populations in the northern half of the north Island (www.nzfrogs.org). Introduction of disease into any of these populations could potentially result in extinct or near extinction. For example, the collapse of the Coromandel *L. archeyi* population which saw the population reduced by 88.0% in 1996 was hypothesised to be the result of Bd entering the population (Bell et al., 2004). Indeed, globally, chytridiomycosis is an example of a disease that drives extinction: a disease that is spread through contact between individuals or contact following brief exposure of infected animals to a water source potentially results in widespread exposure of wild populations to a virulent disease. With 45.0% of amphibian incursions being intercepted post- border the risk of disease transmission would seem high. Furthermore as 88.0% of incursions involved only a single individual, it would be safe to postulate the risk of introducing a disease is higher risk than the risk of establishment of an invasive species into New Zealand.

Interceptions were more likely to occur at the border, n=102 border interceptions compared to n=84 post border interceptions. However, when looking at the numbers of individuals that were intercepted the reverse trend is seen with overall animals detected post border (n=151) than at the border (n=115). This is reflective of the higher numbers of individuals per incursion event that are smuggled in. This points once again to the increased risk of a smuggled amphibian species becoming established in

Upon entering New Zealand's borders, amphibians that were not immediately seized or discovered by biosecurity personnel were transported considerable distances (average of 135 km) once past the border entry point. There were six instances where the incursions were intercepted on a different island from where they initially landed. The longer the time that an incursion goes undetected the greater the chance of disease transmission and dispersal of any disease and the more opportunity it has to enter the local environs. One of the key factors determining where animals are detected is how animals enter: accidentally introduced amphibians are likely to be intercepted at the border whereas smuggled amphibians were always intercepted post border. Smuggled amphibians are at a high risk for both disease and becoming an invasive species as they are transported in larger numbers and are likely to be distributed after entering the country. This has been exemplified with the European newts that were both found to be Bd positive (Laycock et al., 2015) and also found to have a naturalised population (Anonymous, 2015)

Amphibians that were intercepted came from more than 30 countries, and all except three have had confirmed cases of chytridiomycosis. My results confirm that there is a risk of Bd positive amphibians entering via the borders. As such, precautionary measures of handling and disposing of all amphibians intercepted and any damp surface they came in contact with should be sterilized thoroughly with a disinfectant.

Seasonality

The most distinct aspect of seasonality was the difference between alive versus dead amphibians found in winter, with amphibians more likely to be found alive during the winter months June –August and less likely to be found dead. One explanation for this is catch bias: catching live amphibians is easier in the cooler months of winter due to their slower mobility. An alternative explanation could be a higher survival rate as amphibians will have a higher body condition, particularly at the beginning of winter, and a slow metabolism during cooler months therefore increasing their survivability.

It is also important to note that the presence of more live individuals in winter increases risk of spread of Bd as the individuals are more likely to be infected with a high infection load. The optimum temperature for Bd survival is between 17-25^{o}C, hence it could be expected that there would be more deaths in spring, autumn and winter from Bd infection due to the cooler moister climatic conditions (Piotrowski, Annis, & Longcore, 2004). Meanwhile during summer, the hotter drier weather in New Zealand could mean the risk of desiccation is higher. These data did not entirely reflect this with the most deceased individuals being recorded in spring and summer yet winter there were more live individuals. As there was an absence of Bd found in the collection the higher death rate in spring and summer most likely reflects a poorer body condition in spring and desiccation during summer.

Evidence of Bd in the Sample.

As part of this study, I tested for the presence of Bd in the MPI samples. Although there was no clear positive result for Bd in the Anurans, however, seven of the 13 newts tested positive. The newts tested were all fresh samples with less than a month in storage and were euthanised just prior to storage in 70.0% ethanol. However, given the newts are from a naturalised population, it is unclear whether the European newts that founded this population arrived infected with Bd or obtained it post arrival in

New Zealand. The ambiguity of the histological samples was unsurprising as many of the samples were in poor condition.

In 2008 the Organization for Animal Health (OIE) further added to the list of amphibian diseases, both chytridiomycosis and ranavirus to be listed in the Aquatic Code and made notifiable to the OIE. The code goes further and states it must be reported when "diseases listed by the OIE, if the disease has occurred with a new pathogen strain or in a new disease manifestation" (1.2c World Organisation for Animal Health (2008) Diseases listed by the OIE. Chapter 1.2.3, article 1.2.3.4. In: Aquatic Animal Health Code 2008. OIE, Paris).

Yet prior to the present study no Bd disease screening of intercepted amphibians was undertaken. Furthermore, identifying different strains of Bd in New Zealand has historically been limited with only four strains having been identified since 1999. Disease screening at point of entry could prove an economical way to fulfil any screening requirements as search and capture costs of wild animals would be unnecessary.

Conclusion

With ever increasing anthropogenic movement of trade goods and increasing global movement of humans, biosecurity has become a progressively complex process but an increasing necessity. The MPI data on seized amphibians has highlighted several key areas of the increased risk of incursions.

The overall the risk of an accidentally introduced amphibian becoming naturalised or invasive in the New Zealand environs could be considered low as most incursions include low numbers of individuals. However, the dataset does reveal several species that regularly enter the country and these species may pose a substantial risk of becoming naturalised or invasive. The most concerning of these is the cane toad, although it is unlikely to threaten New Zealand's endemic amphibians it would pose a threat due to its toxicity to many endemic and threatened insects, reptiles, and birds. The green tree frog also has the potential to become naturalised but is unlikely to become as invasive or a pest species as, apart from in the northern part of New Zealand, the climate is marginal for its survival. Furthermore, it is susceptible to Bd which is already present in New Zealand. My results show that the naturalisation of smuggled amphibians is a higher risk, of the two smuggled incursion seizures in the collection one has become naturalised.

Disease risk poses a significant and potentially greater threat to New Zealand's endemic amphibian biodiversity than species naturalisations. In relation to Bd infection, there are a regular quantity of potentially Bd infected frogs entering the borders, although Bd is already present in there is a risk of new and more virulent strains entering. Currently, detected incursions of individuals entering New Zealand are not tested for Bd. From the amphibians in the current MPI data set none had undergone any previous disease testing. Nor does it appear that the people involved with capture and disposal of the specimens are being treated as possible disease carriers. Awareness of the potential Bd disease threats by biosecurity personnel is therefore an urgent priority.

Recommendations

There were several areas that could be addressed to help mitigate exotic amphibian introductions and their associated diseases entering New Zealand.

These include:

- identifying the pathways that smuggled amphibian enter the country,
- adjusting surveillance efforts in relation to season and origin,
- modifying shipping pathways to cooler regions of New Zealand where survival of amphibians would be less likely as most of the live interceptions originate from more temperate/tropical climates,
- more education and training in handling and storing any seized animals.

The border security procedures used to ensure that no exotic amphibians have become naturalised in New Zealand since the introduction of The Biosecurity Act 1993 has been relatively successful. Within this data set only one species, the European newt, has evaded border security and become naturalised. A further species, the green tree frog was also found in considerable numbers but was in captivity. Both of these invaders were smuggled. Intercepting smuggled amphibians at the border appears to be a weak point in border security; it is also of high risk for exotic amphibians to become established in the wild. A third case of a suspected smuggled amphibian *Limnodynastes dumerilii*, was discovered in the Waitakare ranges in 1999, interestingly none of these individuals were included in the MPI collection. They were not tested for disease as they were incorrectly not seen as a risk being that the group consisted of tadpole eggs and metamorphs; it was not realised the water that contained them could be contaminated with Bd zoospores (Pers. Com Whitaker 2013). More investment is required to identify and stop smuggling pathways.

I detected seasonal bias in incursions in each hemisphere - therefore greater vigilance in screening northern hemisphere cargo in the spring and southern hemisphere cargo in the autumn might be pertinent. Similarly, the climatic conditions at the New Zealand port of arrival also influence risk: The northern parts of New Zealand are more likely sites for naturalisation events to happen. Redirecting shipping to more southern ports could be an option if incursions became more frequent.

Finally, all seized amphibians should be treated as though they are diseased. Staff involved with incursion responses should have adequate training in disease control and should be able to quickly access a register of the main disease risks. Routine testing of intercepted amphibians for the main known amphibian pathogens like Bd and Ranavirus should be mandatory. If cost is a concern the specimen should be stored to maximise the ability for latter testing. The current method of storing the entire specimen in 70.0% ethanol could be improved to increase disease detection if parts of each specimen were also stored in formalin and frozen. Finally, ongoing disease testing for Bd is important as any future strains that enter the country risk combination with the current strain resulting in a more virulent strain.

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“Public sentiment is everything. With public sentiment
nothing can fail; without It, nothing can succeed”.

Abraham Lincoln

3 Online Sales of *Litoria* spp. in New Zealand.

Abstract

Trade Me™ an online selling platform is a popular site for the sale of a wide variety of things including animals, both farm stock and pets are regularly sold. The introduced species of amphibians are frequently sold via Trade Me™, as it is illegal to keep and sell any of the four endemic *Leiopelma* species of frog. There a six different species of amphibian available from three different genera: *Ambystoma Mexicana* (axolotl), *Cynops orentalis* (Chinese fire bellied newt), *Cynops pyrrhogaster* (Japanese fire bellied newt), and three *Litoria* species *L. aurea* (green and golden bell frog), *L. raniformis* (southern bell frog) and *L. ewingi* (brown tree frog)

Trade Me™ was monitored over a two-year period and all sales of the three *Litoria* species (*L. aurea*, *L. raniformis*, and *L. ewingi*) were recorded with details of the sale. *Litoria* species are mostly wild-caught and easily sent around the country via the New Zealand postal service. These factors make the online sale and movement of them an ideal model to evaluate disease transmission. The exclusion of other amphibians from the data set was because they were captive-bred and wild populations had not been recorded to date in New Zealand. The exception was to record if a seller sold any of the other species.

The results showed that large numbers of *Litoria* were sold and transported widely throughout New Zealand. It was uncommon for stock to be bred specifically for sale, rather it was collected from the wild. With the majority of animals being traded being in the tadpole stage, which is asymptomatic for amphibian chytrid, the risk of spreading this disease throughout the country is high.

Introduction

Frogs and tadpoles are popular childhood pets in New Zealand where all three of the introduced *Litoria spp.* are kept as pets. Furthermore, tadpoles are often found on nature tables in junior classrooms to meet the learning objectives incorporated in New Zealand’s national science curriculum. Specifically, the living world strand of the curriculum, it has the three learning areas of life processes, ecology, and evolution (Aitken, Sinnema, Newman, Kelly, & Kirkus-Lamont, 2007). It is relatively easy to obtain *Litoria spp.* tadpoles and even adult frogs in New Zealand as they are not a protected species and they can be freely gathered from the wild, bought from most pet shops, or from online trading sites such as Trade Me ^{TM}. Once the tadpoles have reached maturity they are often released into the wild. A quick search of the internet supplies many examples of frogs being released or advising to release frogs on maturity https://www.wairakei.school.nz/blog/frogs-in-room-3/ https://www.wikihow.com/Raise-Tadpoles https://www.discoverwildlife.com/how-to/watch-wildlife/how-to-rear-froglets/. It is a common misconception that the release of frogs into the wild is helping the amphibian populations thrive, observed in the websites that advise release. Interestingly, even overseas release programs fail to stress that amphibians should not be released in their advocacy material for example. Although this is not a New Zealand-based program the issues with the spread of Bd are a global problem, as are many other diseases for amphibians, as is disease transmission in general for all species. Advocacy and education to control disease transmission involve public understanding and compliance. Creators of education and advocacy programmes should be aware that the worldwide web is accessible worldwide, and therefore education via this pathway needs to address issues that arise broadly and encompass a worldwide audience.

In New Zealand, although there are no restrictions on gathering *Litoria spp.* from the wild, there is a restriction on releasing them into the wild. The Conservation Act 1987, Section 26ZM, states "No person shall transfer live aquatic life or release live aquatic life into any freshwater". This law is not widely known or enforced so this process of obtaining, keeping, then releasing amphibians poses a high risk of spreading pathogens such as Bd, ranavirus, or any possible new or unknown pathogens.

Anthropogenic movement of animals poses a disease risk to wild animals, especially to endemic and native fauna who may be naive to introduced disease or parasites (Goulson & Hughes, 2015; Williams et al., 2002). Human mediated transportation of animals increases the dispersal of disease in animals as natural biogeographical boundaries are eliminated. Furthermore, rapidity of successful 'dispersal' is greatly increased in the pet trade as buyers. Sellers who distributed large numbers of individuals or sell numerous species provide a channel or bottleneck for anthropogenic movement of animals as numerous animals from different origins and location pass through a single location or facility thus creating the potential for increased contact between animals and thus can be considered 'hotspots' for disease transmission.

Being an isolated island nation, the geographic location of New Zealand makes non-anthropogenic introduction of new species a rarity. This has both been an advantage in that it provides protection against invaders, and a disadvantage as the flora and fauna have high levels of endemism and are highly specialised and therefore vulnerable when invasions do occur. New Zealand's physical geography has also historically provided barriers to species dispersal. There is a natural sea barrier between the two major islands and outlier islands that provides a barrier for the spread of introduced animals and the disease vectors they carry. The anthropogenic movement of animals whether intentional or not negates this natural defence. Therefore, the primary mode of movement for terrestrial animals between the two main islands and their outliers is associated with human activity. Since the arrival of the first humans in the 11th century A.D. the composition of the fauna and flora of the country has changed dramatically. From the first introductions brought by the Māori like *Rattus exalans* (kiori), *Canis lupus familiaris* (kuri), *Ipomoea batatas* (kūmara) and *Lagenaria siceraria* (hue) there has been a constant and rapidly increasing number of introduced species and significant extinctions and range reduction of native species. Modernity has enabled humans both fast and extensive movement between countries and with that the explosion of both intentionally and accidental movement of other species.

In an attempt to reduce the risk of biological invasion and to increase biosecurity aimed at protecting both the economy and environment, New Zealand introduced The Biosecurity Act in 1993. Presently, the legal international trade of amphibians in and out of New Zealand is highly restricted and extremely limited. However, nationally there is little regulation of animals' movement apart from specific incursion events. An example of this would be the recent outbreak of *Mycoplasma bovis*, a bacterial infection that affects cattle. For this specific incursion event MPI put restrictions on the movement of cattle and equipment from infected farms in an attempt to eradicate the disease (https://www.mpi.govt.nz/protection-and-response/mycoplasma-bovis/what-is-mpi-doing/legal-notices-for-mycoplasma-bovis/).

Unless there is a specific outbreak that affect domestic animals either directly or indirectly or a threat to specific endemic fauna or flora there are no overriding preventative measures enforced by MPI. Public can find out about current alerts from the MPI website, (https://www.mpi.govt.nz/protection-and-response/responding/alerts/), other than that it appears information appears to be very targeted to specific audiences directly affected by the incursion/alert. The movement of introduced amphibians nationally is completely unregulated and unmonitored. As a result, there is also little known about the nature of national trade of amphibians within New Zealand. To address this knowledge gap and better understand the potential risks to New Zealand's native amphibian populations, I monitored the online trading of frogs through Trade Me^{TM}, a popular online selling website in New Zealand. Determining the extent of anthropogenic movement of the three introduced *Litoria* species of frog within New Zealand through Trade Me ^{TM} transactions is key to evaluating the disease risk of this activity to New Zealand's endemic amphibians.

Methodology

The New Zealand website Trade Me ^{TM} was monitored for 24 months between 1st Jan 2011 and 31st December 2012. During that time, all sales for frogs and tadpoles were searched for using both manual searches and automated notifications. There were 12 different categories of information recorded: *Trader ID, date of sale, location of seller, location of buyer, type of animal being sold, correct identification of animal being sold where possible, age of animal, number of animals, the availability of more animals, whether husbandry advise was given, whether buyers were told not to release, and if the animals were captive bred or wild caught.* Only listings that resulted in a sale were recorded. All data were entered into an Excel spreadsheet for checking, collating, and analysis.

There were several different ways searches for frog sales were achieved. Searches included both manual searches and automated notifications. Manual searches were searches that used either 'frog' or 'tadpole' as the search word these searches were done daily, and all sales were recorded. Trade Me ^{TM} has an automated listing notification service whereby any new listing with the words frog or tadpole are sent via email to the enquirer. The automated service was utilised in addition to manual searches. Automated notifications are a service Trade Me ^{TM} provides where any listings of a specific requested item will be directly emailed to the enquirer. This method results in any listing with a particular word within the listing being emailed i.e., frog toy, frog keyring etc.

In addition to both the manual and automated searches of the current listings, the sales feedback of specific known amphibian traders was manually searched. This provided an opportunity for any listings that were only online for a brief time period, for example if a buyer requested a listing from a seller, the seller would put it up at a certain agreed time and the sale would go through immediately. Even using both methods, it is likely that the number of sales was underestimated. For example, searching through a particular known seller's feedback there were several instances where additional transactions were discovered. The total number of animals recorded as sold is also a highly conservative number. This is because some sellers advertised tadpoles or frogs individually, whereas in reality the buyer would have purchased multiple animals under the single sale. The likely reason for this behaviour is the avoidance of paying sales success fees to Trade Me ™ as the online company charges a percentage of the successful sales price.

Results

Trade Me ™ allocates all sellers and buyers a general area/location as part of being a member permitted to buy and sell. This allowed all buys and sellers to be assign to a geographic area for the purpose of analysis. There are 18 New Zealand regions that are further divided up by 141 areas, Australia and Chatham Islands are also included: although there were no listings recorded from these last two locations. In total, over the two-year period, 934 transactions were recorded: 55.7% n= 520 were listed by North Island sellers compared to 44.3% n=414 by South Island sellers. In the two years of the study there were differences between years; in 2011 43.1% n= 403 sales compared to 2012 56.9% n= 531 sales. This difference in the number of listings is due to an intra-island difference. Comparison of listings when broken down by year for each island illustrates a lower number of South Island listings in 2011: South Island 2011 33.5% n=135 compared to 52.5% n= 279 in 2012. Meanwhile in the North Island the two years although similar in number of sales (2011 n= 268 and 2012 n=252) the percentage of total sales was different 2011 66.5% and 2012 47.5%. One explanation to this difference between years in the South Island is the devastating earthquake in Christchurch that occurred in February 2011 and likely reduced the sales of amphibians that year. Looking at the monthly sales there is a clear dip in monthly sales in February this also supports this see figure 3.2.

Developmental Stage of Animal at Time of Sale

The age of the animal was recorded as either tadpole, metamorph or adult. Note I have used the following labels to define life stages: tadpole from Gosner stage 20 -41; metamorph Gosner stage 42 – 46; adult is an individual that has completed metamorphosis i.e., when all the Gosner stages have been completed. The sales consisted of 70.0% (n=652) tadpoles, 24.5% (n=225) adults, and 0.5% (n=53) metamorph/larvae.

Location of Sellers and Buyers

In reference to the seller’s location, of the 934 sales listed over the two-year period 520 (55.7%) originated from the North Island and 414 (44.3%) were from the South Island. The North Island human population is 3.3 times larger than the South Island so relatively more amphibian sales per human population occur in the south. This becomes more apparent if sales are looked at yearly; in 2011, the South Island had 33.5% of the sales which reflects the proportion of inhabitants, however, in 2012, 52.5% of the sales originated in the South Island.

During the two-year period there were 832 transactions where the location of the buyer was recorded. In 2011 45.8% (n=381) buyer locations over the total two-year period were recorded, consisting of North Island 60.6% (n=231) and South Island 39.4% (n=150). In 2012, there were 451 buyer locations recorded (North Island 49.7% n= 224 and South Island 50.3% n=227). Amphibian sales were more likely to be outside the seller's local area refer figure 3.1; same area sales accounted for 37.6% (n=313) of sales compared to 62.4% (n=519) of sales between different areas. Frogs and tadpoles were sent between North and South Islands 11.5% n=96 compared to 88.5% n=736 transactions sent within an island. There was a similar flow between islands with north to south transactions 58.3% (n=56) being slightly more frequent than south to north 41.6% n=40.

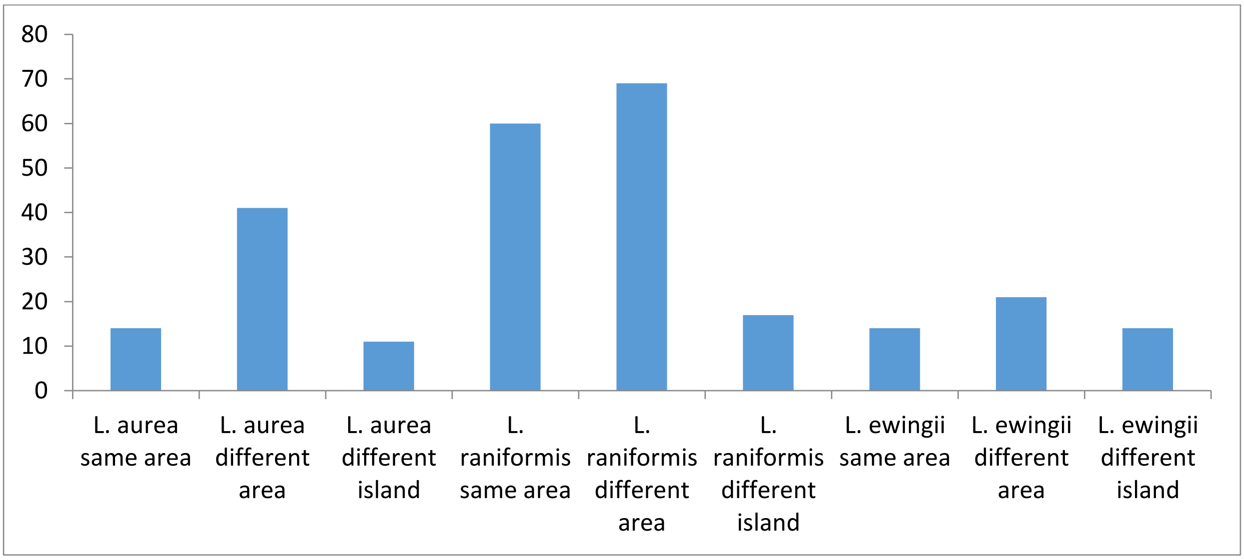

Figure 3.1 Movement of animals: within an area, between areas and between islands by species.

Online Listings and Identification Names Used

Data for all three species of *Litoria* frog were recorded. Identification of the species being sold was complex as there were many incorrect identifications of frogs. Furthermore, the identification of tadpoles of the *Litoria* species is difficult with *L. aurea* and *L. raniformis* being identical to the eye. The names used in listings included a total of 32 different names (Figure 3.2) ranging from scientific nomenclature to common names including unspecific names e.g., 'green pond frog' and even just ‘frog’. The use of taxonomic nomenclature was rare with only 1.3% (n=12) listings using *Litoria* as the genus 11 of these were of *L. aurea* (n=4), *L. aurea* split(n=7), there was a single listing using *Litoria ewingii*. The *L. aurea* listings where from three separate sellers, while the ‘*L. aurea* split’ were from a single seller the split was referring to the animal being heterozygous for an albino gene. Using the common name to identify the animal for sale was ubiquitous, however the names used varied with 32 separate common identifiers used.

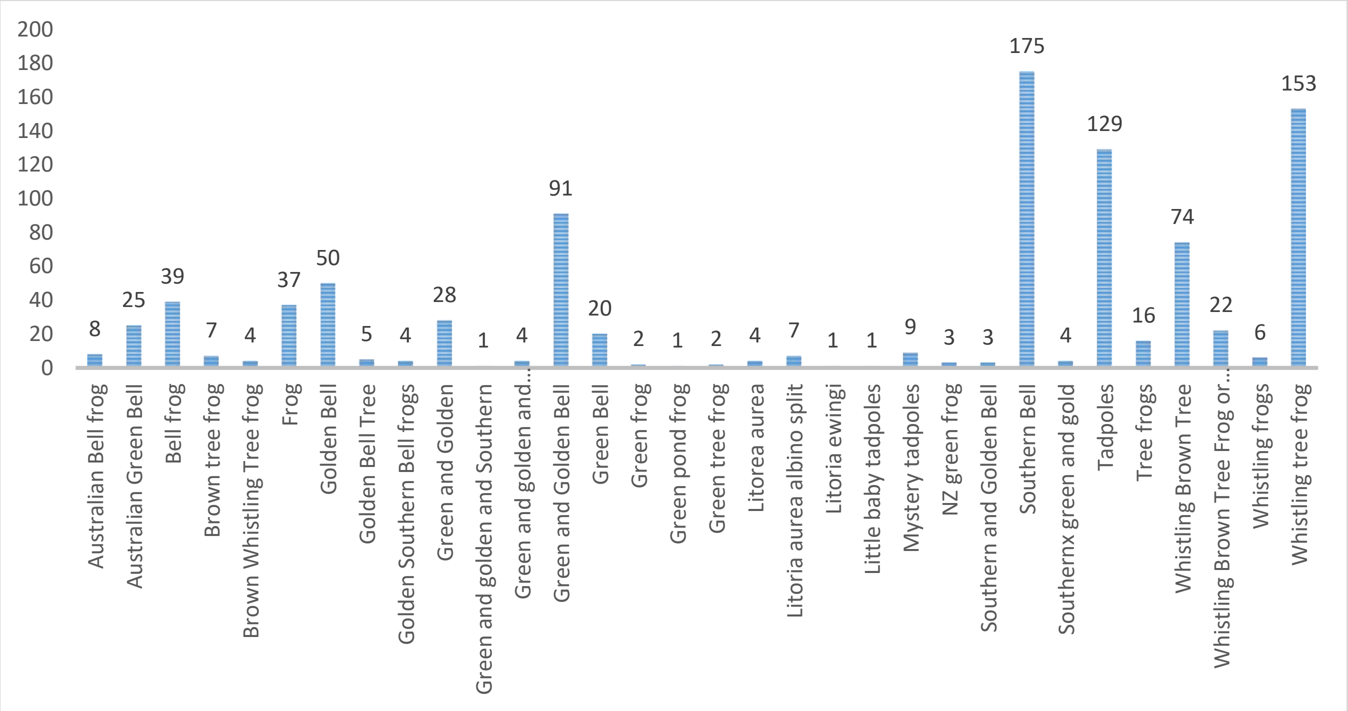

Figure 3.2 Identification of amphibians for sale by sellers.

Accuracy of Identification

Sellers supplied a photo with their listings, but often these are not of the actual animal being sold as sellers either stated not actual animal on the listing or it was apparent that the image had been downloaded from the internet. In many cases it is hard to decipher what species was being sold from the photograph due to the angle or quality of the image. 0f all the listings 26.7% (n=249) listings where a photo ID was possible, of these listings 76.0% (n= 189) were correctly identified. Because of the small size of *Litoria ewingii*, this species was always correctly identified. Conversely *Litoria aurea* was most often incorrectly identified. Most of these listings with ID photographs were for adult frogs 79.9% (n=199) and for tadpoles 20.1% (n=50). Regarding tadpoles’ sales with photographs, only sellers that included photos of adult cohorts or parents were able to be included due to the difficulty of identifying *Litoria* tadpoles.

Species Being Traded

Determining the actual species being traded was problematic for several reasons. Often the name was generic i.e., little green frog and the photo unclear or traders misidentified the species, however 30.4% (n= 284) listings where I could accurately identify the species either by photograph, or information gleaned from description, location and photograph. I also identified by purchasing tadpoles myself n= 140 from 14 different transactions (the tadpoles were bought in cohorts of 10). Of the identifiable animal listings 54.2% (n=154) were correctly listed as *L. raniformis* followed by 27.1% (n=77) *L. aurea* and 18.7% (n=53) *L. ewingii*. These results should be observed with caution, as pointed out previously, *L. ewingii* is more easily identified and therefore data could be skewed towards a greater representation of this species. However, it is unsurprising *L. raniformis* is the most common species sold as it has the largest habitat range of the three species not only covering the entire length of New Zealand but with many populations throughout. *L. ewingii* although found from the far north to the deep south has more patchy population with more consistent population coverage being in the South Island.

Seasonality

There was marked seasonality in the listings of *Litoria spp.* with spring and summer (October through February) having the largest number of sales refer Figure 3.3. This is to be predicted as amphibians are more visible and audible during this these months. All life stages of frogs showed seasonality in sales, although metamorphs were only sold in low numbers refer Figure 3.4.

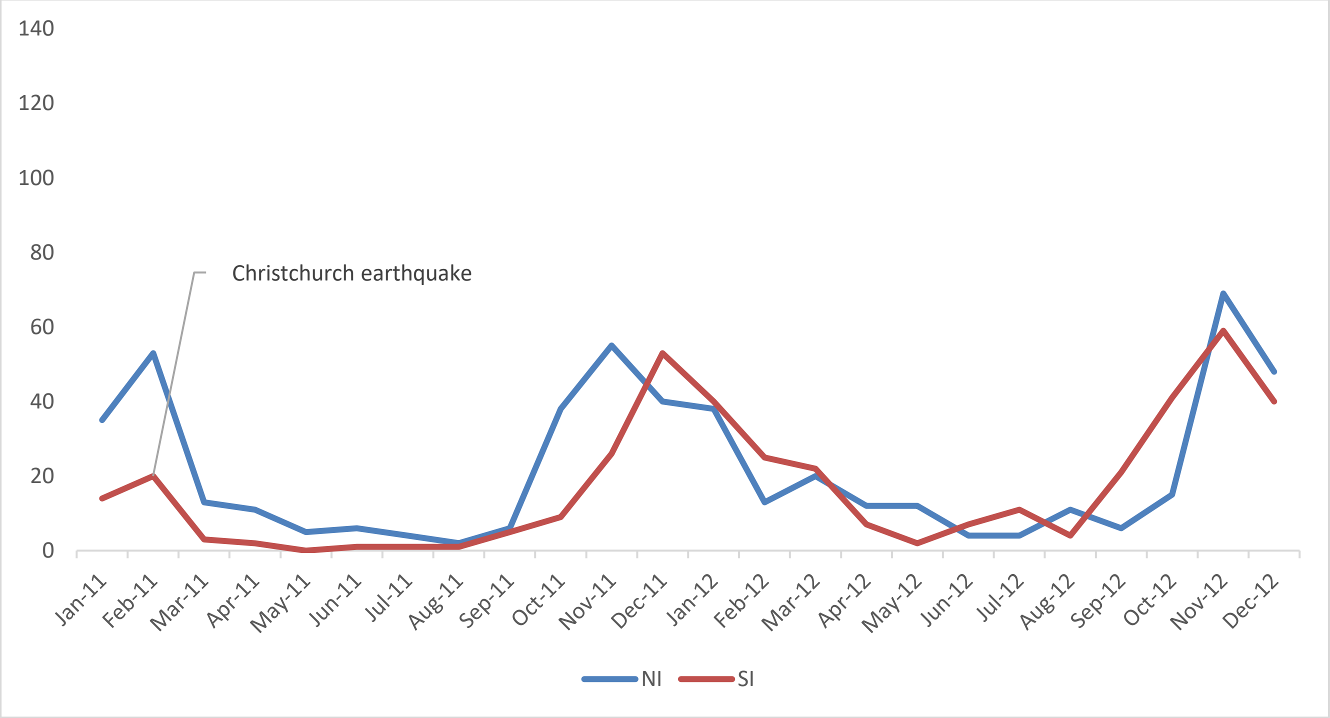

Figure 3.3 Total monthly *Litoria* spp. sales 2011-12 North Island compared to South Island (all life stages and species combined).

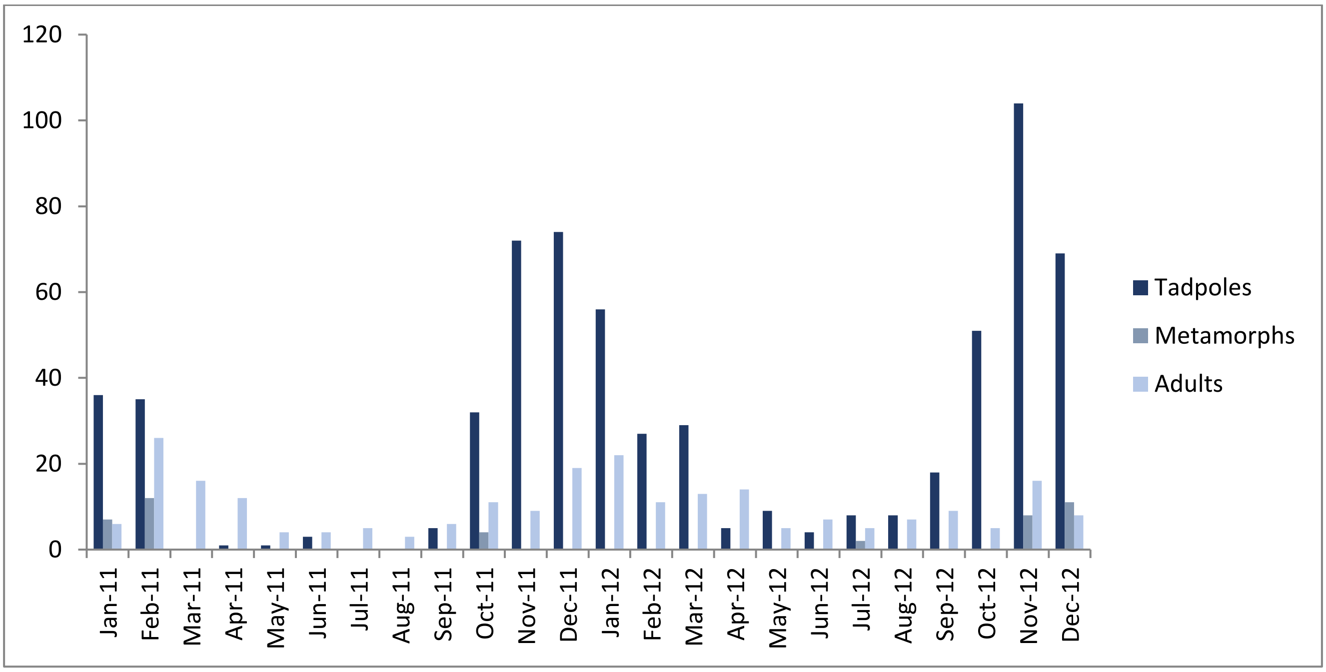

Figure 3.4 Number of *Litoria* spp. sales per month 2011-12 by life stage.

Number of Trades Per Seller and the Number of Animals Per Trade

There was a total of 186 recorded traders over the two years that I monitored Trade Me ™. Of these 55.4% (n=103) of sellers had a single amphibian transaction. There were 44.6% (n=83) traders that had many sales; 10.8% (n=20) traders had >10 sales, with one trader having 169 sales over the two years. The number of animals advertised in each listing varied between one and 200. The most frequent listing was of a single individual (n=234) followed by with listings of 10 individual n=197 and 5 individuals n= 140 (Figure 3.5).

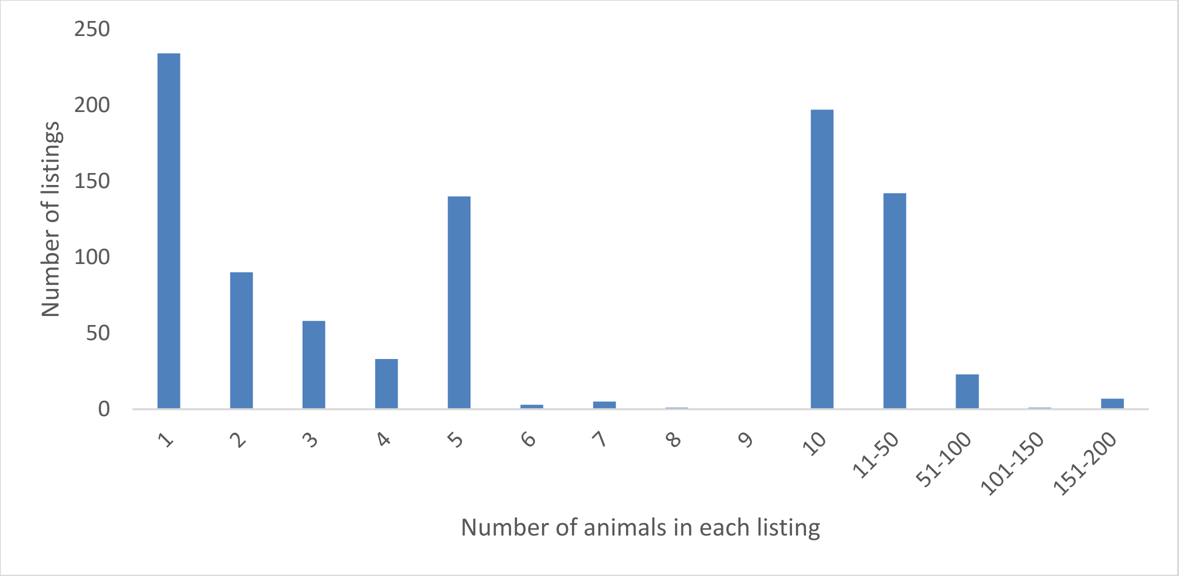

Figure 3.5 The number of animals per listing during 2011-2012.

Sellers frequently offered more animals for sale on a single posting. It is suspected this is common practice used to avoid Trade Me ™ selling fees. Evidence for this assertion comes from the fact that 51.1% (n=477) listings that stated more animals were available compared to 48.9% (n=457) listings where no more animals were offered. Looking at whether single sales were more likely to offer additional non-listed animals to purchase it would appear so. Of the 234 listings that were of a single animal, 74.4% (n= 174) sales were for a single animal with more offered, compared to 25.6% (n=60) sales of a single animal where no more were offered. Comparatively of the 700 listings selling more than one individual 43.3% (n=303) offered more while 56.7% (n=397) did not offer anymore.

Origin of Animals

In New Zealand all three *Litoria spp.* can be freely collected from the wild. The data were divided into four main categories: *captive bred, wild caught, obtained from a pet shop or other trader* and *undefined*. Undefined (meaning the origin of the animal was not stated) was the most common 68.3% (n=638) of listings. It would be safe to assume, because there are no major breeding farms and the animals particularly tadpoles are easy to collect from the wild, that the majority of these would have been collected from the wild. There were 27.9% (n=261) of listings that stated animals came from the wild. There were 3.0% (n=28) listings stating the individuals had been captive born and only 0.6% (n=6) listings stating that the animals had previously been purchased from a pet shop or bought from another trader.

Husbandry and Release Information

Like all living things amphibians have specific needs to survive and thrive. Understanding the needs of a newly acquired animal to ensure its wellbeing would seem important for their care yet 69.3% (n=647) listings provided no advice on how to look after the animals. Brief advice was classified as either giving information on one of the following: food requirements or living conditions or lifespan. Basic information was categorised as giving information in two or more parts of brief advice (i.e. food and living requirements) 16.8% (n=157). Some listings directed buyers to the New Zealand frog website www.nzfrog.org for husbandry information 8.7% (n=81) although this was never to the specific page on husbandry. There were 4.5% (n=42) listings that provided a care sheet with purchase. There was no evidence in any of the transactions that explained the risk of amphibian chytrid and how to stop contamination via equipment and contaminated water. Likewise, there was no guidance given on release for 71.5% (n=668) listings. Out of 934 listings over two years only twice (0.2%) were there listings stating do not release into the wild. Of even more concern is the fact that 28.3% (n=264) listings instructed purchasers that release would be positive in helping re-populate frog-free areas.

Discussion

The international trade in amphibians is vast and includes trade for human consumption, bait, pets, zoo exhibits, bio-control releases and laboratory animals. The trade is largely unregulated and deals with huge quantities of animals and has a high monetary value. For example, the global trade in frogs' legs amounted to over $43 million U.S. dollars in 2005 (Schloegel et al., 2009, 2010). In 2005 over 4.5 million live amphibians were traded across the borders in the U.S. (Ibid.). Personal involved in amphibian trade are often unaware their stock maybe carrying pathogens (Schloegel et al., 2009, 2010). Trade in animals, not only increases disease transmission within a species it also risks human health as zoonoses increase due to increased contact between animals and humans (Gibbs, 2005) .

New Zealand has strict border controls, however, movement of plants and animals within the border is largely unrestricted with the exception of incursion events and the movement of cattle and deer. Incursion responses are essentially very specific time and place events e.g., Queensland fruit fly whereby the movement of fruit and vegetables was restricted in a specific and defined area. The movement of cattle and deer is monitored through the National Animal Identification and Tracing (NAIT) scheme in which all animals are required to be tagged and registered in a national database. The focus of animal and plant movement restrictions within New Zealand is predominantly to ensure New Zealand's primary industries stay pest and disease free. Unlike the strict rules on most native and endemic fauna (with the exception of endemic fish), introduced wildlife is rarely the focus of movement restrictions however all wildlife especially endemic wildlife may benefit from these restrictions. The movement of amphibians throughout New Zealand is significant with a minimum of 11,279 individuals being listed and sold via the Trade Me ™ site over the two years the site was monitored. This number is extremely conservative as many of the listings offered more animals and the sales were often of more individuals than were indicated on the listing. Trade Me ™ encourages feedback from both buyer and seller and reading through this feedback it became apparent that sales often included extra individuals.

Tadpoles were by far the most common life stage at which individuals were sold. This is not surprising given that tadpoles are easy to transport via posting. Animals are readily sent in water in a plastic bag or plastic bottle, or jar placed in a box and posted. For amphibians, the tadpole stage is also the stage that poses the highest risk for disease transmission of Bd, as tadpoles are asymptomatic (Smith & Weldon, 2007). This would increase the ability of the disease to spread considerably as sellers could unwittingly be selling diseased but asymptomatic tadpoles. As it is common practise to collect stock from the wild it would be hard to tell whether stock was infected or not.

The distance of the movement of animals is another disease risk factor. Cunningham (1996, pp. 352) noted in regard to translocations to mitigate disease risk it is advisable to "Maintain the animals in captivity as near to the site of capture/release as possible". The same principle could be applied to the movement of pet amphibians: the shorter distance from source they are moved the less risk of spreading disease into naive populations. The Trade Me ™ website is exacerbating the anthropogenic movement of amphibians as most sales are not local; with many sales being shipped between Islands.

Trade Me ^{TM} has aided the movement of amphibians far more than previous methods of natural migration or the childhood hobby of local collection keeping and releasing tadpoles and frogs. Allowing rapid dispersion of an introduced species with no regulation and minimal information about care and release. Regarding amphibians, this research suggests the postal and courier service has been a major contributor to the translocation of both tadpoles and frogs in New Zealand.

The lack of husbandry advice including hygiene protocols of keeping equipment clean and free from Bd contamination and disposing of wastewater were absent from all listings. Only two listings of the 934 recorded stated not to release frogs/tadpoles into the wild. This is of major concern as it will intensify the normal rate of disease transmission. It is not uncommon for people to release sick or ailing tadpoles and frogs thinking they will be better off released into the wild. The purchase of tadpoles in some instances were specifically to release. Although the three *Litoria spp*. that have established wild populations in New Zealand are widespread and generally not seen as a direct competitive threat to the endemic *Leiopelma spp*., this is largely a result of different habitat requirements. Nonetheless, the risk of disease transmission is likely but the extent unknown. All three introduced species have been tested positive in New Zealand for Bd (Shaw et al., 2013); the first record of Bd was discovered in *L. raniformis* in 1999 (Bishop, 2000; Waldman et al., 2001). Bd in turn has been implicated in the 1990's crash of the *L. archeyi* population in Coromandel (Bell et al., 2004). Although Shaw et al 2003 found at least adult *L. archeyi* showed some resistance to Bd infection. This needs further investigation as it has been documented that as tadpoles' complete metamorphosis they can be more susceptible to the effects of Bd infection (A, 2017; Cohen et al., 2019; Fernández-Loras, Fernández-Beaskoetxea, Arriero, Fisher, & Bosch, 2017; Rollins-Smith, Ramsey, Pask, Reinert, & Woodhams, 2011).

Chytridiomycosis is an evolving disease with several strains already present in New Zealand, although it appears these strains appear to originate from the BdGPL strain from Asia (Sumpter, Butler, & Poulter, 2018). The introduction or the evolution of a new virulent strain of Bd could be a major threat to *L. archeyii* and *L. hochstetteri* as these two species are found on the mainland of the North Island. Aside from the risk of the newly introduced or evolved virulent strains, the reinfection of habitats with different strains of Bd could also be a risk. Jenkinson et al. (2018) notes that virulence, production of zoospores and host mortality are not necessarily directly correlated as has previously been. Furthermore, it is unclear the risks of increased disease virulence and effects of amphibians in habitats that are being repeatedly infected with different strains of Bd. As such avoidance of reinfection would be advisable.

Any releases of *Litoria spp.* into the wild is not only of concern for disease transmission. The three *Litoria spp.* that have naturalised in New Zealand are all terrestrial frogs and are considered generalist feeders (Pyke & White, 2001; Pyke, 2002). *L. raniformis* the most widespread of the three species have reportedly been seen to prey upon invertebrates, lizards, snakes, and small fish (Ayers, 1995; Martin & Littlejohn, 1982). Similarly, *L. aurea* has been observed to prey on many species including lizards, crickets, cockroaches, dragonflies, grasshoppers, caterpillars, slugs, and earthworms to name a few (Pyke & White, 2001).

Like most amphibians *Litoria spp*. are considered opportunistic feeders and the size of the prey is more relevant than species of prey: the size of gape being the restriction (Browne, 2009; Pyke & White, 2001). Although they would not compete with or threaten *Leiopelma spp*. they have the potential to threaten our many endangered reptiles and invertebrates. *Litoria* frogs hunt both diurnally and nocturnally (Barker & Grigg, 1977; Barker et al., 1995) many of New Zealand's endemic reptiles and invertebrates are renowned for being nocturnal so would be vulnerable to predation.

The movement or relocation of *Litoria spp*. into novel or ecologically sensitive areas is likely to happen for several reasons. Firstly, both the frogs and tadpoles are easily transported, even over long distances via postal service. The *Litoria* species are quite mobile frogs and *L. aurea* can travel up to 10km (Pyke & White, 2001) although they are more likely to stay within a kilometre of their home site. Given that most of the New Zealand has a climate that is both relatively mild and damp (https://niwa.co.nz/education-and-training/schools/resources/climate/overview), the year-round movement of *Litoria spp*. is to be expected in many areas. However, the three species would pose different risks. Both *L. aurea* and *L. raniformis* are large frogs and therefore have a larger gape resulting in the ability to eat a large range of prey. Both *L. aurea* and *L. raniformis* are more temperature sensitive than *L. ewingi*. *L. aurea* prefers warmer temperatures, mainly being found above latitude 39^{o} south above Gisborne (Bishop, 2008). *L. raniformis* although found throughout New Zealand has been observed to become more torpid in lower temperatures (Cree, 1984) and therefore would only migrate/disperse during the warmer months. *Litoria ewingii* is found throughout New Zealand it is more arboreal than the other two species and can cope with much cooler temperatures including freezing temperatures (Bishop, 2008; Cree, 1984; Rexer-Huber et al., 2015).

Animal Welfare Issues

In New Zealand the introduced *Litoria* species of amphibians are not given any protection under New Zealand"s Wildlife Act (New Zealand Government, 1953). As such they are freely collected, kept as pets, transported throughout the country and largely released. Apart from pet shops one of the most common ways to acquire tadpoles and frogs is through Trade Me^{TM}. Trade Me^{TM} is a New Zealand online selling platform that can be used to sell a wide variety of items including animals. Narayan and Hero (2011) have a set of listing guidelines https://help.trademe.co.nz/hc/en-us/articles/360011223951-Listing-animals and a Trade Me^{TM} Animal Code of Welfare https://help.trademe.co.nz/hc/en-us/articles/360018831911. The animal code of welfare pertains mostly to dogs and cats and possibly more broadly and vaguely to all animals. The listing guidelines state unlike cats, dogs and other animals including livestock and horses, fish, mice, rats, chinchillas, bearded dragons, and turtles can only be auctioned. Amphibians are freely sold on Trade Me^{TM} with very little restriction, to the extent they are not even mentioned in the under policies and restrictions of selling animals. Amphibians are sold through auctions rather than classified adverts, as such are sold to the highest bidder meaning there is no screening of the buyer suitability.

The transportation of amphibians when purchased through Trade Me ^{TM} is commonly via the New Zealand postal service. Tadpoles are sent either in a plastic bag or bottle, frogs are sent in a container with some damp medium such as sphagnum moss. Delivery time ranges from overnight to three working days and longer if the address is rural. It should be noted that the overnight service is by courier. New Zealand post prohibit the sending of animals excluding correctly packaged bees, leeches, silkworms, and harmless insects (https://www.nzpost.co.nz/personal/sending-within-nz/prohibited-restricted-items#prohibited). This clearly is not strictly enforced, possibly because tadpoles may be assumed not to fit into the animal classification, or senders do not identify what the parcel contains. Amphibians are not listed in the exempt animals so senders may either not be aware of posting restrictions or assume they do not fall under the category of animal. Further to the legalities of sending amphibians via post, it is questionable whether to do so is actually humane. The postal service would have no training, and neither would they have any controls in place to ensure the safe handling of amphibians due to them being a prohibited item. The handling of the package containing the amphibians is paramount in reducing stress of the animals. For example, Swallow et al., (2005) advise the temperature should remain within the range the animal is accustomed to and extremes should be avoided. This may be hard to achieve if sent via post or courier. Several studies have measured the stress hormone in adult frogs that were transported either from the wild or between facilities and noted an increase in corticosterone levels indicating transportation was stressful (Holmes et al., 2018; Narayan & Hero, 2011). It should be noted that in these studies the transport was carried out by experienced amphibian handlers. Holmes et al. (2018) used three different types of substrates in the containers to measure different stress levels in relation to substrate all three invoked a stress response suggesting other factors were triggering the response. There are several known stressors to amphibians that occur during transportation. These have been shown to have various stress outcomes. These range from the release of stress hormones, aggression, loss of condition and cannibalism (Cikanek et al., 2014; Davis & Maerz 2011; Holmes et al., 2018).

Known stress triggers that may occur during transportation include vibration, novel housing, novel social groups, temperature changes, crowding and handling (Narayan & Hero, 2011). All of these are likely to happen whether the amphibians are being transported via post courier or collected privately, albeit times in transit may vary. Increase in stress induces the release of corticotropin resulting in several physiological stress responses including impaired immune responses (Rollins-Smith, 2017). Impaired immune response will result in either a higher infection load and or susceptibility to get infected.

Like all living things amphibians have specific needs to keep them healthy and to survive. The lack of husbandry information provided by sellers was a concern as was the process of Trade Me^{TM} as a platform for selling with its lack of regulation. Online selling of animals in general needs specific guidelines and regulations that need to be monitored. In the case of auctions this would be hard to achieve. It should be noted that some animals on Trade Me^{TM} are not permitted to be auctioned: cats, dogs and horses. Like amphibians, rodents, reptiles, birds, fish, and farming livestock can all be put up for auction and sold to the highest bidder.

During metamorphosis tadpoles undergo a complete reorganisation of the immune system in preparation for adult life and a move from water dwelling to a more terrestrial life (Rollins-Smith, 1998). During this stage and early adult life, individuals are more susceptible to disease (Bakar et al., 2016; Rollins-Smith, 1998). It is also during this stage they are most likely to release into the wild having been kept in most cases to observe metamorphosis. This has several concerning implications. Young adults with their suppressed immunocompetence if infected with Bd could not only have a higher infection load that will result in low chance of survival, but with the stress of translocation into a new environment survival would be even more unlikely. If the individuals are not infected with Bd and released at early adult stage, they are more likely to be susceptible to Bd in the environment and become infected. The release of host species (vulnerable amphibians) into a Bd contaminated environment would help maintain Bd in the population at that specific location.

Conclusions

The online sale of amphibians poses a considerable disease risk due to the number and frequency of amphibians involved and distance they are transported. Regarding Bd, the highest risk would be transmission to naïve populations of *Litoria spp*. and re-infecting areas that are currently Bd free as the *Litoria* spp. are more widespread than the endemic *Leiopelma* spp.. The constant movement of Bd infected individuals around the country is risky because of the threat of creating a more virulent strain is a high risk, Greenspan et al. (2018) state Bd hybrids were more virulent and resulting in a higher mortality rate in hosts than either of the Bd parents. Jenkinson et al. (2018) found that anthropogenic movement of pathogens, via infected frogs/tadpoles, resulted in competitive pressure and this could "rapidly alter the genetics, community dynamics and spatial epidemiology of pathogens in the wild" (Greenspan et al., 2018). The chance of Bd being passed directly from the introduced *Litoria* frogs to the endemic *Leiopelma* frogs would be lower as the two genera do not generally live sympatrically. Only *L. archeyi* and *L. hochstetteri* live on the mainland of the North Island with *L. archeyi* being found throughout the Coromandel Peninsula and in Whareorino forest where it lives sympatrically with *L. hochstetteri.* Meanwhile, the more widespread *L. hochstetteri* is found as far north as Waipu with populations found in patches spreading south in Brynderwyn, Omaha, Mahurangi, Waitakeri, Hunua, Maungtautari, Otawa, Rangitoto, and in the East Coast Raukumara (Phillip J Bishop et al., 2013). *L. archeyi* tend to live in forested areas under rocks and logs, and the semi-aquatic *L. hochstetteri* habitat is forested stream. Meanwhile the *Litoria spp*. found in New Zealand tend to be inhabit grasslands, farmland, and disturbed sites that have a water source nearby (Bishop, 2008). However, in the Whareorino forest there was a sighting of *L. aurea* within the area inhabited by *L. archeyi* and *L. hochstetteri* there was also evidence of predation on Leiopelmatids by *L. aurea* (Thurley & Bell, 1994). It is unclear how widespread sympatric inhabiting between *Litoria* and *Leiopelma* is, although it is interesting to note that in the Whareorino forest site the *Leiopelma* frogs were also found in more open grassland sites, as well as more heavily forested areas (Thurley & Bell, 1994).

The presence of a reservoir species that either moved between the two niches could pose a threat to spreading Bd into *Leiopelma* populations although there is at least some evidence adult Leiopelma spp. have at least some resistance or ability to self-cure from Bd infection (Ohmer, Herber, Speare, & Bishop, 2013; Shaw et al., 2010). Alternatively, and probably more likely would be the anthropogenic spread via contaminated equipment or clothing entering Bd free areas as spores can last up to seven weeks in water (Johnson M. L. & Speare R., 2003) so any damp equipment or water, moist soil or damp fomites could be contaminated.

Predation of endemic species by *Litoria* spp. in New Zealand is largely under reported. Currently there are no published data on stomach contents of *Litoria* frogs in New Zealand apart from the above Whareorino site where it was observed a single *L. aurea* individual was recorded to have *Leiopelma* hindlegs in its stomach (Thurley & Bell, 1994). All three species of *Litoria* are noted to be opportunistic feeders of both vertebrates and invertebrates including members of its own species (Pyke, 2002; Pyke & White, 2001). New Zealand has many threatened and endangered reptiles and an unknown number of threatened invertebrates, many of which could be prey for the opportunistic *Litoria* spp.

New Zealand's endemic *Leiopelma spp*. of frogs are stringently protected, it is illegal to collect or keep them as pets, all four species are endangered to some level with *L. archeyi* being critically endangered. All are under a recovery plan that states as one of its objectives "Public support and community awareness of native frog conservation is increased throughout the term of this plan" (Bishop et al., 2013, p. 12). Public engagement in native *Leiopelma* frog is complex as not only are they not permitted to be kept by the public, they are nocturnal, slow moving, cryptically coloured, do not call, are scarce and live deep in the forest. Conservation success of species relies on both funding and public engagement. Herein lies the problem with amphibians in New Zealand: the need to keep the public engaged with amphibians yet the endemic species are rarely seen by the public. Schultz (2011) notes education is not enough to motivate people to engage in conservation, people also need connection i.e., need to be close to the problem, as well as the issues and conservation effort being a social norm: widely understood. In reference to frogs in New Zealand this would be hard to achieve due to the inaccessibility of the endemic frogs. However, the unprotected introduced *Litoria* frogs could provide such a conservation advocacy opportunity. In effect the more people participate in keeping, caring, and understanding the widespread and accessible *Litoria* frogs the more they would be interested in and motivated to help conservation efforts of *Leiopelma spp*. This of course is a double-edged sword as the more people engage in frogs the more, they will be moved around and possibly released. There also the risk of enthusiastic froggers setting their sights on keeping *Leiopelma spp.*

On one level, interest in *Litoria* frogs has achieved this interest and concern for frogs: the sales that stated 'suitable for release' were an attempt to repopulate frog-less areas, comments posted on Trade Me^{TM} often mentioning the lack of frogs in areas that they used to be abundant. The other issue that became apparent was the confusion over *L. ewingii*, with several comments stating it was one of the endemic *Leiopelma* frogs. The confusion between *L. ewingii* and the endemic *Leiopelma* by the public, was also noted in the NZ frog recovery plan (Bishop et al., 2013)

The risks and benefits of online sales need to be assessed. The juxtaposition of the availability of amphibians as pets and endemic frog conservation is a tenuous one for conservation. The development of a solid guideline on frog husbandry and ownership is imperative. There is an excellent comprehensive New Zealand-based website (www.nzfrogs.org.nz) that provides information about husbandry ownership and the plight of the endemic New Zealand frogs. However, this resource seems to be underutilised by sellers online with only 82 of the 934 listings either mentioning it or linking to it.

Recommendations for Online Sale of Amphibians

To reduce the movement of amphibians all sales should be pick up/collect only. This will keep species within a limited boundary and limit both disease transmission and the spread of populations into new areas. Online sales should not be auctions, they should be a fixed price so sellers can have more control over who purchases their stock. All buyers should complete a simple online questionnaire with a few key questions about husbandry and the responsibilities of owing amphibians when viewing any amphibian sales. Information on the best way to travel or move frogs and the responsibilities involved with this needs to be emphasised. Travelling or moving frogs or tadpoles is stressful for them and guidelines to reduce this stress need to be easily accessible as it is a key animal welfare issue.

Each listing should have a link to the www.nzfrogs.org.nz website and buyers should be encouraged to visit it regularly. The New Zealand frog website is an excellent resource and contains a lot of information especially pertaining to the endemic *Leiopelma* frogs. It contains many resources for teachers as well as interesting videos and merchandise. The website was first developed in 2006 so at the time of writing it has been operating for 14 years. It may be beneficial to reassess the efficacy of the website in getting key amphibian conservation messages across. Currently, it appears the focus is on education about New Zealand's endemic frogs with other information available if searched for. If as suggested above and in the 2015 NZ Frog Recovery Plan that *Litoria* frogs be used to engage the public in the plight of New Zealand's endemic frogs, the design of the website may need refocusing or a new or linked website developed that could focus on this. Key objectives should include husbandry, welfare, and a clear message not to release and why.

The goal of an advocacy programme should not only be to inform, change opinions and impart knowledge of a species predicament, ultimately a positive action or behaviour change that is beneficial for conservation should be sought. “Effective solutions to environmental problems require the active participation of scientifically and technologically literate citizens” (Brewer, 2002, p. 577). It has been well documented that bottom-up conservation is both a necessary and effective strategy in the recovery of species (Brechin & West, 1990; Sodhi et al., 2011; Tsiripidis et al., 2018). However, merely providing information about a particular species is often not the most effective way to engage and motivate people to change their behaviours to more ecologically sustainable ones. “Providing factual information about the animals and making the public aware of environmental problems are considered only initial steps to the larger objective which is persuading audience members to take personal action by changing their behaviours in ways that can make a difference in the survival of wildlife” (Yerke & Burns, 1993, p. 366). Research suggests emotion or affective connection is a stronger motivator than knowledge and if knowledge is used alongside affect there will more likely be a behavioural change (Hodak, 2008).

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4 Amphibian Pet Shop Sales

Abstract

Freshwater vertebrates are sold throughout New Zealand via pet shops, with many pet shops specialising specifically in freshwater vertebrates and invertebrates rather than mammals and birds. The *Litoria* species of tadpole are regularly sold as pets along with frogs and other amphibian species such as Chinese newts (*Cynops orentalis*), Japanese newts (*Cynops pyrrhogaster*), and Axolotl *Ambystoma mexicana*. Bringing different species of amphibians together provides a high-risk opportunity for disease transmission.

Pet shop ownership is largely unregulated with anyone able to buy or set up a shop in New Zealand. To mitigate the risk of spreading disease this study investigated husbandry practices, numbers of amphibians being sold, and source of stock being sold. Furthermore, basic knowledge of amphibians in New Zealand was assessed.

Results showed high numbers of amphibians are sold each year via pet shops, with tadpoles particularly being sold in very high numbers. Pet shop husbandry practices were varied and often inadequate to prevent the transmission of disease. Finally, the general knowledge of the legalities and knowledge of New Zealand's endemic amphibians was generally poor.

Introduction

Pet ownership in New Zealand has not been systematically investigated, resulting in limited available data (Gates MC, Walker J, Zito S, & Dale A, 2019a). Concerning my research, no specific information on the extent of amphibians as pets in New Zealand could be found in a literature search. A search of google scholar using keywords pets, animal companions, amphibians, pet ownership, and New Zealand was undertaken four times during 2020. The only information about amphibian ownership in New Zealand was gleaned from Fifield and Forsyth (1999). However, it was not very informative about numbers as they categorised frogs under 'other' along with reptiles, axolotls, fallow deer, and snails. Not only were the frog's part of the 'other' category this category was the smallest category of animals that families owned: consisting of 2.5% of the total of species owned by families (Dembicki & Anderson, 1996; Fifield S J & Forsyth D K, 1999; Ogechi et al., 2016).

Pet ownership has often been associated with positive health outcomes (Beck & Meyers, 1996; Friedmann & Thomas, 1995; Lewis, Krägeloh, & Shepherd, 2009). Although the link between health benefits and pet ownership has been dismissed by some research (Fraser G et al., 2020; Parslow & Jorm, 2003; Parslow, Jorm, Christensen, Rodgers, & Jacomb, 2005). However, most studies either focused on dogs and cats, or these two species made up the bulk of the data. (Dembicki & Anderson, 1996; Fraser G et al., 2020; Lewis et al., 2009; Ogechi et al., 2016). Yet amphibians are sold through the pet trade a vast number (Herrel & Meijden, 2014; Schloegel et al., 2009; Wombwell et al., 2016). People keep amphibians for many reasons and observation of the metamorphosis from frog to tadpole is probably a primary reason although other reasons include novelty, body shape, and call (G. M. Burghardt, 2017; Measey et al., 2019). Keeping amphibians to observe metamorphosis is included in the curriculum of junior classes globally (Measey et al., 2019).

In New Zealand, the three introduced Australian frogs (*Litoria raniformis, L. aurea,* and *L. ewingii)* are all kept as pets to varying degrees as there are no restrictions on collecting, keeping, and distributing or selling them. In contrast, the four *Leiopelma* species endemic to New Zealand are all strictly protected, and it is not permitted to keep or collect them without a Department of Conservation permit. Pet shops around New Zealand frequently stock tadpoles of the Australian frogs during the spring when the tadpoles are found in abundance in the wild. To date, New Zealand has no known large commercial breeders of *Litoria* frogs for the pet trade, although there are a couple of hobbyists who breed small numbers of amphibians to sell (personal observation). These enthusiasts tend to breed for leucitic appearance traits and do not breed in large numbers and did not supply any of the pet shops included in this research although they did sell via the Trade Me^{TM} website.

It is now widely accepted that animals are sentient beings (Bousfield B & Brown R, 2010; Unknown, 2013). In New Zealand the Animal Welfare Act defines any mammal, bird, reptile, amphibian, fish, and any other aquatic as an animal (Unknown, 2013) https://www.legislation.govt.nz/act/public/1999/0142/latest/DLM49664.html. It has the scope to add to this definition by adding the clause iv “(vii) any other member of the animal kingdom which is declared from time to time by the Governor-General, by Order in Council, to be an animal for the purposes of this Act” (p. 11). The development of the five freedoms by The Farm Animal Welfare Council and John Webster in the 1990s (Bousfield B & Brown R, 2010; Mellor DJ, 2016) has been internationally accepted as best practice for all animals by most professional groups including veterinarians, various SPCA groups worldwide, and the World Organisation for Animal Health.

The most common domestic pets in New Zealand are dogs and cats (Fifield & Forsyth, 1999; Fraser G et al., 2020). Overall New Zealanders are aware of conservation issues and there has been a considerable effort and expenditure in educating the public about conservation issues New Zealand's endemic flora and fauna face (Seabrook-Davidson MNH & Brunton DH, 2014; Towns DR, Daugherty CH, Broome K, Timmins S, & Clout M, 2019). However, although there appears to be a general awareness of conservation issues by the public, this awareness does not correlate to positive behaviour changes of domestic pet owners that will ensure endemic fauna are not threatened (Gates MC, Walker J, Zito S, & Dale A, 2019b; Woolley CK & Hartley S, 2019).

"In New Zealand, the owner of an animal and every person in charge of an animal is obliged to ensure that the animals needs are met in a manner that is in accordance with good practice and scientific knowledge" Animal Welfare in New Zealand (Unknown, 1999), p. 41). This means all New Zealanders animal owners should understand the needs of the animal and meet the animal welfare obligations for that animal (Unknown, 2013).

Unlike mammals' amphibians do not use facial expressions to display emotions (G. M. Burghardt, 2017). They still experience stress and suffer if their needs are not met (G. M. Burghardt, 2017; Warwick et al., 2017). Because both amphibians don't use anthropomorphic methods of communication, owners of amphibians and reptiles need to be aware of stress signals to ensure that animals are not suffering. These include aggression, hyperactivity, hypoactivity and interaction with transparent boundaries, there are at least 30 stress behaviours that have been identified in reptiles (Warwick et al., 2017). It would be expected a similar number in amphibians. Overseas studies have reported high mortality rates, abuse, and neglect in the amphibian trade (Ashley et al., 2014; Warwick et al., 2017). It has been reported that in the United Kingdom around 75.0% of all reptiles kept domestically die within the first year (Ashley et al., 2014). Both reptiles and amphibians have complex biological needs that need to be met if they are to have fulfilling lives (Gordon M Burghardt, 2013).

To reduce the threats to native biodiversity, the pet industry in New Zealand needs to ensure it addresses three key issues: reducing the risk of spreading of invasive and or exotic animals, mitigation of the spread of disease and animal welfare. To achieve this, we require an understanding of the risks each species poses if released, what diseases are associated with the species being sold, and clear guidelines about the welfare needs of each species that must be understood and clearly communicated to any future owner.

Methods

To evaluate the extent of amphibian sales, pet shop husbandry practises, educational material provided, and the amphibian knowledge of New Zealand pet retailers, a questionnaire was taken to pet shops in three of the four main cities during the beginning of 2011. Christchurch pet shops were not included due to the devastating earthquake that happened in February 2011. Several small-town pet shops were also given questionnaires. One of the largest pet shop chains in New Zealand was asked to take part but declined and shortly after stopped sales of amphibians in all their branches throughout New Zealand. Included in the participants were a museum curator from a museum who displayed *Litoria* frogs and amphibian enthusiasts who either collected or bred *Litoria* frogs and sold them via the internet.

The data was entered onto an Excel spreadsheet and analysed by descriptive analysis. Human ethics was not required as it was considered a low-risk project as the questionnaire was not invasive and only given to adult participants. Furthermore, no pet shops or participants included in the study were identified.

Twelve questionnaires were undertaken and included pet shops (n=8) amphibian enthusiasts (n=3) museum (n=1). A meeting time was arranged with the owner or seller of the amphibians. The participants were given an information sheet (Appendix 7.1) explaining the projects and outlining the responsibilities of the researcher and the obligations of the participant. (Appendix 7.3). Each participant was asked to sign a consent form prior to taking part in the research (Appendix 7.2). During this meeting, the participants were asked to complete the questionnaire (see appendix) in the presence of myself: the researcher. The main reason for the researcher being present was the short eleven-question general knowledge test at the end of the questionnaire. The aim was to evaluate the participant's current knowledge so participants could not search for the answers to the questions and to clarify any queries the participant may have. The questionnaires were completely anonymous: participants could not be identified in relation to a particular questionnaire after it was completed. The questionnaires took approximately 20 minutes to complete. Once completed they were taken back to Massey University and entered into a spreadsheet.

Results

The respondents that formed the basis for this study varied considerably in the type of association they had with amphibians, hence not all questions in the questionnaire were applicable to all respondents. For example, museum staff did not sell amphibians, and many amateur enthusiasts sold stock collected from the wild rather than breeding stock. Nonetheless, some general patterns emerged; the most popular species to keep or sell was *L. aurea* with 75.0% of respondents either selling or holding them in collections. *L. aurea* tadpoles were the most common stage/species to be sold, with numbers greater than 2000 being sold between nine sellers. Fewer, but still significant, numbers of *L. aurea* frogs were sold at lower numbers with just under 450 being sold per annum by five sellers. Axolotl (*Ambystoma mexicana*) were the next most common species to be available for sale with 67.0% of respondents holding them and approximately 600 being sold each year. Forty-two percent of respondents kept *L. raniformis* and more than 750 tadpoles were sold each year between four sellers compared to less than 250 adult frogs being sold by three sellers. *Litoria ewingii* was stocked by 42.0% of respondents with around 350 tadpoles being sold each year between three sellers and less than 100 frogs being sold by two sellers. Unknown tadpoles were stocked by 33.0% (n=4) of respondents with c 250 being sold each year by four sellers. Both Chinese newts (*Cynops orentalis*) and Japanese newts (*Cynops pyrrhogaster*) were each sold by 25.0% (n=3) of sellers, but both species were sold in low numbers (< 150) annually. Finally, *unknown* frogs were sold by 17.0% (n=2) and were comprised of <100 individuals per year.

Pet shops sold the widest range of species with these organisations supplying between two to eight species. Amphibian enthusiasts usually focused on a single species with just one of these respondents having two species in addition to tadpoles of unknown species. There were two pet shops that sold unknown frogs, and surprisingly, one of the pet shops that indicated in the questionnaire that they always correctly identified stock, asked me to identify one of the frogs they were selling. The frog was clearly *L. raniformis* and not only did it exhibit typical physical characteristics of the species, but it was also sourced locally from a wild population in the South Island. Under these circumstances, identification of this frog should have been straightforward, as *L. raniformis* is the only species of green frog found in large populations in the wild in the South Island (Bishop, 2008).

Identification of tadpoles is more difficult and, unsurprisingly, the species of tadpoles being sold to pet shops were identified only 50.0% (n=4) of the time by the suppliers. Alternatively, if the supplier did not identify the species 50.0% of pet shops, some pet shops ‘guessed’ and two pet shops said they used morphological features to identify. Both *L. aurea* and *L. raniformis* are almost morphologically identical as tadpoles, so are extremely hard to identify in this way.

Participants were asked whether they kept records of where they obtained their stock. This question was specific to pet shops and was recorded for each species they sold. There were four categories 1 = no records, 2 = name and number of the supplier, 3 = full details of the supplier, and 4 = full details of the supplier and the location of where the stock was obtained. Of the 39 responses from frog and tadpole species, 74.0% had at least some records while 26.0% had no record of where the stock was sourced. In these cases, the animals were simply bought into the shop by someone who had caught them in the wild. Conversely, with both axolotl and newts, the details of suppliers were always recorded.

When asked about the procedure of introducing new stock, most respondents either just placed new stock straight in with existing stock (33.0%) or quarantined, then placed stock in with existing stock (33.0%). Two respondents put stock into a new aquarium. One respondent quarantined then put into a new aquarium and one quarantined then put the tadpoles into a new sterilised aquarium.
Most (66.7%, n=8) of the tanks that contained stock where individually filtered. There were two respondents who used small group filtration that consisted of several tanks and one pet shop who used a single filtration system for all tanks. There was one amphibian enthusiast who bred/kept their stock in outside ponds. Of the two respondents who group filtered tanks, one was respondent stated that they filtered based on the position of the tanks and the other by what species of stock it held.
When asked about the disposal of wastewater from the tanks, 66.7% (n=8) poured the water straight down the drain, one filtered the water then poured it down the drain. For the other two respondents, one pumped wastewater onto the lawn and the other use the water to water their plants. The participants were asked to rank from one (low) to five (high) what they considered important when purchasing stock. There were eight respondents with one respondent only giving their most important consideration. In order of importance were choosing suppliers who provide the healthiest stock, using the same supplier as previously, choosing a supplier who can provide all the species required, choosing the supplier who gives the best deal, and, of least importance, choosing the nearest supplier.
No specific amphibian equipment (buckets, nets gloves, etc) was found for 50.0% of the ten respondents i.e., they did not have equipment designated to specific tanks/animals. Three respondents said they had designated equipment for each tank (however, it should be noted that this was not observed (during a visit) in one pet shop that had indicated otherwise). One respondent stated they designated equipment for each species and the final pet shop designated equipment based on whether the stock was tropical or cold water.

Most participants stated they cleaned their equipment daily (n=4) or two times a week (n=3). Single respondents either cleaned after each use, weekly or monthly. Of the ten participants who answered how they cleaned their equipment, half-used some form of disinfectant, three rinsed everything and let it dry in the sun, one washed with hot water and soap, and one rinsed under running water until debris was not visible. Of the participants who did keep mortality records (n=5) as opposed to the ones who kept none (n=6), five recorded which species died. Both the date and cause of death was recorded by four respondents each. In addition, three participants recorded the number of dead and a single person recorded which tank the dead came from.

The most common method of disposal of dead animals was putting them in the rubbish bin (n=5) followed by putting in the biohazard waste (n=2), feeding to fish (n=2), composting (n=1), and flushing down the drain (n=1). No biohazard disposal was observed at any of the shops.

Of the ten sellers who responded to the advice section, eight sellers always gave verbal advice when a sale was made while two never gave verbal advice. The written advice was never given by four sellers, rarely by a single seller, half of the time by three sellers, and all the time by two. Eight sellers provided support for all species, while two only provide it for some species. After purchase support was provided by eight sellers all the time. One seller never, and one seller rarely, provided after purchase support. Of the sellers who provided after purchase support, six provided verbal support, two provided both written and verbal, and a single seller-provided written only. All who provided post-purchase support provided it for all species of amphibians that they sold.

Participants were asked to rank their own knowledge from one (limited) to five (expert). Nine of the respondents rated their knowledge at three (average) two people rated their knowledge four (slightly above average) and one person two (slightly below average). When asked if they knew how many frog species were currently in New Zealand eight said yes and four said no. Of the eight who responded yes, only two responded with the correct number of seven. Answers three (n=1), four (n=2), six (n=1).

In the final part of the questionnaire, the participants were asked 11 true/false questions. When asked about endemic and introduced frogs, all 12 participants understood that there were both endemic and introduced frogs in New Zealand. Most (n=9) people understood you need permission to collect some species of frog. Yet, when asked whether anyone could collect frogs and tadpoles from the wild only 58.3% (n=7) answered false, the correct answer. When asked if all species of frogs can be kept in New Zealand, 66.7% (n=8) correctly answered false. The same percentage of people answered correctly with false the question: frogs are not protected by any laws. There was uncertainty related to the release of frogs and tadpoles. Only 33.3% of people were aware that you could not release frogs and tadpoles into the wild. The same percentage correctly thought that you could not release them even into the same place they were gathered. Only 25.0% of respondents were aware it is illegal to release frogs and tadpoles into the wild in New Zealand. Furthermore, only 25.0% of people knew you would not hear or see New Zealand's endemic *Leiopelma* frogs in ponds. Finally, pet shop owners were verbally asked about where they obtain their stock. There were two main sources: the most common being the people who supply their cold-water fish. The other smaller source was people who bought them in casually. All stock was caught from the wild: none were intentionally bred specifically for the pet market. The cold-water fish breeders sold tadpoles as an aside as they were found in their outside breeding ponds.

Discussion.

The use of questionnaires and their validity to assess behaviours has problems as people tend to answer to meet the social norm of a situation. (Clausen, 1968; Karp & Brockington, 2005; Lelkes, Krosnick, Marx, Judd, & Park, 2012; Sjöström & Holst, 2002). Social desirability when answering the questions in this questionnaire were apparent even though it was stressed to participants there was no wrong or right answer. With that in mind, the responses to this questionnaire could be considered best case scenarios as participants most likely responded to what they considered 'best practice'.

Pet Ownership

Previous literature reports low numbers of amphibians kept as pets in New Zealand compared to other pets such as cats and dogs (Fifield & Forsyth, 1999; Fraser G et al., 2020; Lewis et al., 2009). Yet pet shops report selling thousands of tadpoles each year. As a comparison, in New Zealand the number of registered dogs as of 31 May 2019 was 565,757 (http://www.localcouncils.govt.nz/lgip.nsf/wpg_url/Profiles-Local-Government-Statistical-Overview-Registered-Dogs-and-Owners). With the average lifespan of a domestic dog being 10-13 years, dependant on breed and size of the dog. (https://en.wikipedia.org/wiki/Aging_in_dogs) and cats 10-15 years (https://www.vetmed.ucdavis.edu/sites/g/files/dgvnsk491/files/inline-files/Cats-Indoors_or_Outdoors.pdf) similar to that of a *Litoria* frog 15 years (http://www.nzfrogs.org/Resources/Kids+Information/Keeping+Frogs.html).

Records show approximately 8000 pure bred puppies are registered each year in New Zealand (http://www.fci.be/en/statistics/ByYear.aspx?year=2018 , https://www.nzherald.co.nz/lifestyle/the-most-popular-dogs-in-new-zealand/R4JTSSUTPOP3GNARCMNKR3ASKQ/). Dog registration statistics show 63.0% of registered dogs are purebred while 27.0% are crossbred. If the numbers of puppies bred each year are extrapolated from these data, it could be estimated there would be approximately 13,000 puppies bred each year. If we compare this to the number of tadpoles sold via pet shops, the current data suggests over 20,000 individuals, the number outweighs dog sales and these data do not include internet sales of tadpoles. So, if frog ownership is indeed low and the sales of tadpoles is high, where are all the tadpoles/frogs going? The very high attrition rate could be for several reasons: an extremely high mortality rate so few tadpoles make it to adulthood, the tadpoles are not kept as permanent pets and released, or a combination of both. A likely scenario is that frogs and tadpoles are not viewed as long-term pets but more of an educational tool or interest for children as they are relatively inexpensive to buy and keep. Hence, they are easily discarded once metamorphosis is complete. Furthermore, it is not widely known that it is illegal to release animals into New Zealand's waterways. Both a high mortality rate and release are concerning for amphibian welfare. A high mortality rate could be due to amphibian welfare needs not being met resulting in unnecessary deaths. Additionally, it could be due to diseased stock being sold; this particularly relates to the Bd as tadpoles are largely asymptomatic with death occurring during later stages of metamorphosis and early adulthood (Marantelli, Berger, Speare, & Keegan, 2004; Rollins-Smith, 1998). High mortality associated with Bd during the latter part of metamorphosis could result in owners releasing dead or dying frogs into waterways, under the assumption it would be kinder to release them or not wanting to deal with sick animals or not understanding the risk of releasing sick animals into the environment. An additional consideration regarding the release of tadpoles and frogs into the wild are the animal welfare issues. The habitat may not meet the requirements for survival: inadequate food, lack of refuges. In the case of *L. aurea* the most commonly sold species the release of this species in the cooler regions of New Zealand would make survival unlikely. Releasing animals that have been kept captive may cause them undue stress (Englefield, Blackman, Starling, & McGreevy, 2019).

General Knowledge about Amphibians

Overall, the knowledge about anurans was poor, especially in respect to the legal requirements surrounding release; most respondents were unaware that releasing into the wild was illegal. There was confusion about the number of endemic frogs that are found in New Zealand, where you would find them, and how to identify them. Considering most sellers only provided verbal advice regarding frogs it would be safe to assume this advice would only refer to basic husbandry requirements such as food and minimal information on tank requirements. None of the shops had a specific pamphlet with advice recorded. This meant any information given would be reliant on the seller's knowledge and the purchasers ability to retain the instruction from memory.

Husbandry Practices

There were several high-risk areas identified when looking at husbandry practices in amphibian care. Most retailers put new stock in with the existing stock. This practice increases the chance of disease transmission as the infected stock could encounter healthy individuals or stock infected with different diseases or strains of the disease live in close proximity. As the Bd zoospores have a free-living stage in the water the risk of contamination is high. Furthermore, adding new stock to existing stock risks individuals experiencing a stress response as population dynamics change. The density of stock has a profound effect on both the growth rate and timing of metamorphosis (Semlitsch & Caldwell, 1982). Fluctuating populations would be expected to cause density stress.

The other area of concern is the practice of pouring wastewater down the drain. As Bd zoospores can survive in water for up to five weeks, this practice poses a risk of releasing Bd into the environment. Cleaning and designation of equipment provided another area of disease transmission as half the participants reported they did not have designated equipment for each tank meaning water-borne diseases could easily be transferred between tanks. Furthermore, cleaning the equipment varied between stores with half the respondents stating they did not use disinfectant and the majority of sellers did not sterilise after use, rather daily or even weekly. Cleaning methods varied widely with half the stores not using a disinfectant to clean equipment. Some respondents reported they let equipment dry in the sun. This maybe viable in the summer however wintertime would not provide adequate drying times to eliminate chytrid zoospores.

Species to species disease risk.

The risk of disease transmission via pet shops is high, especially in pet shops that also stock newts and axolotl as these species share similar disease profiles albeit the morbidity may differ between species. Newts and axolotl are examples of species that can carry a disease - in this case amphibian chytrid fungus – yet not succumb to it: they are asymptomatic carriers (Bell, 2016; Frías-Alvarez et al., 2008). Infected individuals that are asymptomatic carry disease undetected, in the context of pet shops this could become problematic. The bottleneck a pet shop provides, with many species coming together briefly before being distributed (sold) affords a scenario for increased disease risk or increased contact with a variety of viruses, bacteria, super bugs, or new virulent strains. As a result, pet-shops can act as super spreader venues for disease transmission. These diseases may not only be a risk to wildlife but humans as well.

Zoonoses

The latest deadly zoonosis to have severely affected human health and welfare is COVID-19. The COVID-19 pandemic has resulted in more than two million deaths by the beginning of 2021. It has had an unprecedented effect on most aspects of human society from mental health (Xiong et al., 2020), suicide (Sher, 2020), the economy (Debata, Patnaik, & Mishra, 2020) worldwide there is a rush to assess the damages (Gautam & Hens, 2020). COVID-19 is the third coronavirus to affect humans and is by far the worst, the other two SARS-Cov and MERS-Cov each had under 1000 deaths. All three are zoonoses originating from animals (Wong et al., 2020). Amphibians have several diseases that can affect humans or have the potential to (Pauwels & Pantchev, 2018). There recently has been a case of amphibian-type *Brucella* jumping the evolutionary boundaries and infecting an exotic amphibian keeper (Rouzic et al., 2020). Although New Zealand has yet to report an amphibian disease crossing over to infect humans, the risk remains, and protocols should be designed and actioned to mitigate and reduce the likelihood.

An example would be *Mycobacterium marinum* a bacteria found in freshwater and marine aquatic animals and occasionally humans (Collins, Grange, Noble, & Yates, 1985). Amphibians are susceptible to several species of *Mycobacterium*, (Martinho & Heatley, 2012) report *M. marinum* is the most common species that is present in amphibians. Transmission of *M. marinum* in amphibians is via direct contact with an infected individual, contaminated water, and fomites (Martinho & Heatley, 2012). It has been postulated that *M. marinum* is highly prevalent in aquarium water (Petrini, 2006).

In New Zealand it has been identified in captive pinnipeds and farmed domestic cows (Chatterton et al., 2020). It has also been reported of an infection in a Pacific Island male living in New Zealand (Kevern, Tovaranonte, Meyer, & Pithie, 2014). Clearly *M. marinum* is present in New Zealand, it is unclear if it is present in the pet-trade as to date there have been no published reports on its presence in this sector.

Disease Pathways

Pet shops and Trade Me™ sellers pose contrasting disease risk factors in the amphibian pet trade. Any movement of animals increases the of disease transmission. The disease pathways differ between pet shops sales and Trade Me™ sales. Trade Me™ sellers collected tadpoles from their local environment and posted them nationwide. They mostly only traded in a single species. Conversely pet shops obtained their stock from multiple sources where suppliers were not local. Their clientele would most likely be locals. Pet shops also traded in many species. Between these two methods of trading, disease pathways are intensified: pet shops bring in many species from many sources and distribute them to single locations (e.g., local homes). Conversely, 'Trade Me^{TM}' sellers collect frogs from local areas and distribute them nationally (many locations).

Recommendations

There are two primary targets for education, namely the pet shops (sellers) and the public (buyers). The development of an information pamphlet for amphibian traders giving guidelines about best practice in the care of amphibians and outlining risk areas for disease. This would be a start towards informing and encouraging better understanding both about the needs of amphibians and how to prevent disease transmission. This could be followed up by a yearly interactive online quiz, with possible spot prizes to encourage participation. Alternatively, free advertising on New Zealand amphibian or pet web sites for high scoring pet shop participants, promoting the shop as responsible sellers.

Pet shop owners should be encouraged to source amphibian stock locally wherever possible especially tadpoles and frogs that are more likely to be released into the environment. To help stop the release of frogs and tadpoles, signage informing customers against the release should be clearly visible on all tanks containing amphibian stock.

The other audience to educate are the public, in particular those who purchase amphibians as pets. Education should include husbandry requirements but also life span, general frog fact, explanation of why releasing amphibians into the wild is damaging to both other frogs and the individuals being released. Developing an understanding why amphibians should not be released rather than just stating the legalities about release would more likely have a positive outcome. The inclusion of fun websites and online blogs about amphibians in any educational material with the aim to encourage a sense of community. Furthermore, a yearly online quiz with spot prizes to encourage participation could be used to educate the public both about pet amphibians but also the plight of the endemic *Leiopelma* species.

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5 Identify the presence, transmission, and maintenance of Bd in New Zealand amphibians

Abstract

The global spread of amphibian chytrid fungus has been implicated in the decline, extirpations, and extinctions of many species of amphibians. First identified in New Zealand in 1999 amphibian chytrid is now widespread. Amphibian chytrid was first described in 1998 after mass die-offs at sites in both Australia and Panama. Caused by the fungus *Batrachochytrium dendrobatidis* (Bd) amphibian chytrid provides an ideal model to investigate how a pathogen is spread and then maintained in susceptible populations. It provides an interesting model in part due to the different morbidity outcomes between species and over life stages, and because amphibians are easily transported meaning that disease transmission could be more rapid. The global anthropogenic movement of amphibians through the pet and food trade is vast, although New Zealand is largely excluded from this due to strict biosecurity. However, despite the fact, there are strict border controls and surveillance, there are regular incursion events of alien fauna, flora, and disease, amphibian chytrid being one of these.

The disease pathway of Bd into New Zealand is unclear, it is assumed it was introduced via the pet trade. Transmission has certainly been aided via the anthropogenic movement through the pet trade and it is now found throughout New Zealand in wild populations. The results of this research support previous research that Bd is found throughout New Zealand. Furthermore, this study shows that populations of *Litoria aurea* can successfully survive with Bd at a relatively high prevalence (50%) in the population. Whether this is due to increase immunity resulting in disease resistance to Bd or environmental factors is unclear and needs further investigation. One observation identified in this study was the presence of an asymptomatic species (alpine newt) living sympatrically with *L. aurea.* The presence of a reservoir species appeared to adversely affect the survivorship of the *L. aurea* population. It was unclear whether this was a result of predation or the newts acting as a disease reservoir that kept the Bd infection load too high for the *L. aurea* to survive, or it was a combination of both. The site consisted of few adults and many small individuals who had just completed metamorphosis, so this suggests it was Bd that was responsible for the low numbers of metamorphs surviving to adulthood.

Introduction

The amphibian disease chytridiomycosis caused by the fungus *Batrachochytrium dendrobatidis* has affected amphibians worldwide and is seen as a contributing factor in the unprecedented decline in amphibians. The International Union for Conservation **(**ICUN) states that amphibians are the most threatened group of vertebrates globally; currently, 41.0% of amphibian species are listed as at some level of threatened status ICUN (2021). Reasons for the decline include habitat loss, climate change, disease, pet/wildlife/food trade, alien species, pollution and chemical contamination (Beebee T.J.C & Griffiths R.A, 2005; Bridges C.M & Semlitsch R.D, 2000; J.P., M.L., & T.E., 2009; McMenamin S.K, Hadly E.A, & Wright C.K, 2008).

The chytridiomycota are a group of zoosporic fungi that are phylogenetically related to real fungi (Barr, 2001). They exist in a wide range of habitats and are saprophytes that obtain nourishment from plant matter. There are also parasitic chytrids that parasitise anything from plants bacteria invertebrates and vertebrates (Gsell A.S, de Senerpont Domis L.N, van Donk E, & Ibelings B.W, 2013). The two *Batrachochytrium* species, *Batrachochytrium dendrobatidis* (Bd) and *Batrachochytrium salamandrivorans* (Bsal) parasitise amphibians in the anuran and salamander families respectively. Both Bd and Bsal are considered aquatic chytrids as their lifecycle requires water in the zoospore stage. The zoospores are unwalled mostly spherical cells with a flagellum that gives them motility in water. In the second lifecycle stage the motile zoospores encyst into the hosts epidermis where they reabsorb the flagellum and grow a cell wall. The encysted zoospore develops into a thallus shaped sporangium. The thallus which becomes swollen with developing zoospores is now called a zoosporangium. In the final stages of development the zoospores within the zoosporangia become motile and finally burst out of the zoosporagium via a single tube (Berger L, Hyatt AD, Speare R, & Longcore J.E, 2005). Death of the host occurs because the reproductive zoosporangia live in the keratinised layers of amphibian skin eventually causing hyperplasia and hyperkeratosis, with mortality in susceptible species due to osmotic imbalance resulting in cardiac arrest (Voyles et al., 2007).

Both Bd and Bsal require water or moist conditions to survive. Bd can survive for 8 weeks or more outside the hosts body (IUCN 2005). Bsal has been recorded to survive in water for 31 days at 15^{o}C (Health et al., 2018). Bd will not survive desiccation (Johnson M. L. & Speare R., 2003); at least part of its lifecycle (zoospore stage) is aquatic; during this stage the zoospores are motile meaning one mode of disease transmission is through contaminated water. In moist environments Bd zoospores are resilient and can survive and remain infective in water for up to seven weeks (Johnson M. L. & Speare R., 2003).

Amphibian chytrid fungus is susceptible to desiccation and high temperatures. There is variation in temperature tolerance between Bd and Bsal. When grown in the laboratory *B. dendrobatidis* optimal growth rate occurs between 17-25°C and is killed at 28°C (Longcore J.E., Pessier A.P., & Nichols D.K., 1999; Stevenson L.A. et al., 2013; Van Sluys M. et al., 2008). Meanwhile, *B. salamandrivorans* grew at temperatures as low as 5 °C, with optimal growth between 10°C and 15°C. (Martel et al., 2013) which is considerably lower.

Aside from Bd's parasitic qualities and its prolonged viability in moist conditions there are examples of amphibians acting as reservoirs and vectors, these include *Rana catesbeiana* (Mazzoni et al., 2003); (Schloegel et al., 2009), *Xenopus* spp.. (Weldon C, Du Preez L.H, Hyatt A.D, Muller R, & Speare R, 2004) and *Ambystoma tigrinum* (Annis, Dastoor, Ziel, Daszak, & Longcore, 2004). All appear to exist with sub-clinical infections. Another amphibian that shows no signs of the disease yet can test positive for the pathogen is the Axolotl (*Ambystoma mexicanum*) (Van de Koppel & Vos, 2013). Axolotls are commonly sold throughout New Zealand in pet stores where infected individuals would be a source of infection to other amphibians if strict hygiene protocols are not followed. The extent to which other aquatic animals are carriers of Bd is unclear.

Tadpoles can also provide a mode of transmission of Bd in the absence of adult frogs. As tadpole's skin does not contain keratinised cells they don't appear to be as affected by Bd in their pre-metamorphic state. Tadpoles carry Bd in their mouthparts as this is the only keratinised tissues in their pre-metamorphic bodies (Daszak et al., 1999) "Bd infection frequently caused substantial mortality in post-metamorphs, and mostly sub-lethal, but not-insignificant, effects in tadpoles." (Kilpatrick, Briggs, & Daszak, 2010), p. 113). This would make tadpoles ideal dispersers of Bd in New Zealand as they are readily transported around the country and may show no clinical signs of Bd infection. Translocation of apparently healthy tadpoles by members of the public and by the pet trade has spread Bd to new localities in New Zealand (Waldman et al., 2001).

First discovered in New Zealand in 1999 in a pond near Christchurch (Waldman B, 2001) Bd has been implicated in the decline in *L. archeyi* in the Coromandel ranges ((Ben D. Bell, Carver, Mitchell, & Pledger, 2004) although this has been questioned by (Ohmer, Herber, Speare, & Bishop, 2013; Stephanie D Shaw et al., 2010). It is unclear how Bd arrived in New Zealand although it has been postulated that it may have arrived via the exotic aquarium fish trade (Waldman et al., 2001).

The current research aim was to determine prevalence of Bd in four different types of population: wild populations of naturalised exotic species, the pet trade, a group of invasive individuals being eradicated after a recently discovered incursion event and confinement facilities such as laboratory/zoo populations.

Sample

Sampling included individuals bought via the Trade Me™ Website (*Litoria aurea* and *L. raniformis*), pet shops (*L. aurea* and *L. ewingii*), Auckland Zoo (*L. aurea, Xenopus laevis*, *Ambystoma mexicanum* and *Cynops pyrrhogaster*), the University of Otago (*X. laevis*) and the wild (*L. aurea* and *L. raniformis*). The species tested were a result of what was available or held at each facility or location.

The wild samples consisted of tadpoles that were collected and brought back to Massey University or adult frogs that were swabbed in situ and released. The tadpoles that were caught and brought to Massey university to complete metamorphosis, were caught at various locations around the greater Auckland region. Additionally, included in this study were adult *L. aurea* that were part of a bigger study being undertaken by the University of Newcastle, were sampled over two breeding seasons from two separate sites. Only the data relative to Bd prevalence were included in this research. The larger study aimed to investigate environmental parameters that affect Bd.

Trade Me™ and pet shop individuals were all tadpoles. The Trade Me™ tadpoles were bought via the website and posted via New Zealand Post and subsequently kept at Massey University until metamorphosis was completed. The pet shop tadpoles were collected by me and brought back to Massey University to complete metamorphosis.

The containment facility group data were from individuals held by Auckland Zoo and the University of Otago. These individuals were all adults and kept in stable cohorts and swabbed and immediately returned to the original enclosure on site.

Finally, the individuals from an incursion event of 102 European alpine newts *Ichthyosaura alpestris* that were discovered in the eastern Waikato region in 2013 were tested. This group of individuals had been illegally smuggled into New Zealand and released, becoming naturalised in a localised undisclosed area of the Waikato.

Collected and bought tadpoles were kept until metamorphosis was complete. The tadpoles were kept in a secure building and a strict hygiene routine was followed to avoid cross contamination. Each cohort was housed separately and had separate cleaning equipment. New sterile latex gloves were used whenever cleaning or handling. All cleaning and handling were carried out in a clockwise rotation around the room. Once the entire cohort had completed full metamorphoses each individual was swabbed then the cohort euthanised as required by animal ethics. A cohort consisted of 10 individuals when bought from Trade Me$^{\text{TM}}$ or a pet shop.

With the Auckland Zoo and the University of Otago individuals a cohort was a group of animals living in the same tank.

In this portion of the research a total of 375 individuals were tested for the presence/absence of Bd using standard RT- PCR, consisting of Trade Me$^{\text{TM}}$ n=130, Pet shop n=31, Auckland Zoo n=31, the University of Otago n=10 and wild caught n=71 and alpine newts n=102.

In addition to the above, 439 captures of *L. aurea* adults were tested using quantitative PCR, the PCR was performed by the University of Newcastle researchers. These individuals were swabbed and released in the field at two locations: a Buddhist Monastery at Pukekohe and Ocean Beach. The testing was carried out over two separate time periods during 2014: January, February then again in December. The *L. aurea* individuals were measured, pit tagged and swabbed prior to being released. In total 826 individual PCR tests were performed.

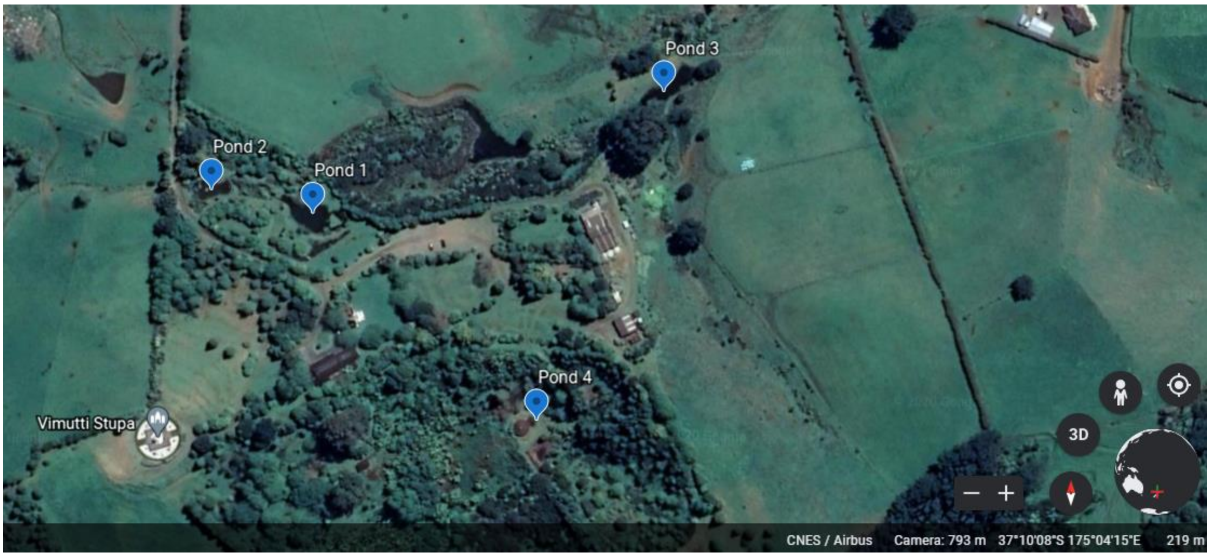

Figure 5.1 Buddhist Monastery at Pukekohe Site

Figure 5.2 Ocean beach site

The reason both RT-PCR and quantitative PCR where used was, the initial research for my thesis just required the recording of presence/absence of Bd in the amphibians tested to record the prevalence of Bd. When I was approached to be part of the larger study by researchers from the University of Newcastle the requirement for that research meant they chose to use quantitative PCR.

The two sites were different in the surrounding vegetation. The sites at Ocean beach were close to the ocean and were more open. Four ponds were sampled in this location with ponds three and four being effluent ponds for the dairy farm they were on. These ponds measured 30mx20m each and were surrounded by grass the other main species immediately surrounding the ponds was Toi Toi (pampus grass). The other two ponds at this location were on a neighbouring property approximately 600 metres away. These ponds where smaller (7mx5m each) and were surrounded by vegetation consisting of reeds and various shrubs. One pond was covered in a species of lily.

The monastery site was inland and had extensive planting of native vegetation over the site. The four ponds from this location had various amounts of vegetation cover. Pond one measured 40mx6m and was predominantly surrounded by grass with a few shrubs 10.0% and trees 5.0%. Pond two was 20mx6m and covered in lily pads over 90.0% of the surface, the edges were covered with reeds, flax, and shrubs. Pond three measured 50mx6m and was mainly surrounded by grass with about 10.0% flax coverage around the edge. Pond four was approximately 18mx15m and surrounded by grass, reeds, and flax.

Ethics approval was obtained through Massey University Ethics Committee **MUAEC Protocol 11/66**

Methods

Each amphibian was swabbed following the protocol laid out in (Hyatt et al., 2007); in summary, the swab was rotated and wiped four times along each side of the animal from beneath the forelimb to the top of the hind limb; four times medially and laterally on each thigh and finally four times on the plantar side of each hand and foot. The swabbing was done with a standard MW100 swab; a standard swab used when swabbing for chytridiomycosis on live or recently deceased amphibians. The swabs were labelled and subsequently frozen at -20ºC. Once the collection of all samples had been completed the swabs were taken to Manaaki Whenua Landcare Research to be processed.

The *L. aurea* swabs collected during the two 2014 field trips to Whangarei and Pukekohe were taken back to the University of Newcastle and processed there using quantitative PCR.

The samples processed at Manaaki Whenua Landcare Research using RT-PCR used the following protocols. The DNA was extracted using Prepman™. Each swab was placed in a sterile Eppendorf ™ and 50ul of Prepman™ was added. The sample was vortexed for 30 seconds and spun down at 8000 rpm for 30 seconds then placed in a thermocycler for ten minutes at 100ºC. After cooling the sample was centrifuged for one minute at 13000rpm and aliquoted into a new sterile Eppendorf then frozen at -20ºC.

Each 15µl PCR reaction consisted of 7.5 µl Taqman PCR Mix 1 µl of Bd primer mix 1.5 µl MGB probe (2.5 µM) and 5 µl DNA. The Bd primer mix consisted of 13.5 µM each of ITS1-3 Chytr and 5.8S Chytr. The DNA was diluted to 1:10 and was pooled into triplicates. The PCR run was as follows: Step 1 Hold at 50ºC for 2 mins, Step 2 hold at 95ºC for 10mins this was cycled 50 times, followed by 95ºC for 15 seconds then 60ºC for 1 min. The samples were run in triplicates. Any samples that were inhibited were rerun at a 1:10 dilution.

Results

The data can be divided into two separate categories due to the type of PCR testing: one data set was tested using standard RT-PCR and the other set was tested using quantitative PCR. It should be noted that the standard PCR results all fall with the standard curve for each reaction while the quantitative PCR results include all positive results including the ones that fall outside the reaction standards.

The Samples Using Standard PCR

This group of individuals was tested in triplicates as the aim was to identify the presence of Bd in each cohort rather than the prevalence of Bd in a cohort. The data collected from amphibians via the Trade Me^{TM} Website, pet shops, Auckland Zoo, the University of Otago, and the wild was tested for absence or presence of Bd in these groups, meaning each cohort was tested for the presence of Bd rather than individuals to determine a positive Bd score. The results are as follows: the Auckland Zoo individuals which consisted of *L. aurea, Cynops pyrrhogaster*, *Xenopus laevis* and *Ambystoma mexicanum* all tested negative for Bd. The University of Otago sample that consisted of *X. laevis* were also negative for Bd infection. Amphibians from four pet shops were tested. The three cohorts of *L. aurea* and one cohort of *L. ewingii* were all negative for Bd.

Of the 13 Trade Me^{TM} cohorts tested three came back positive which were all *L. aurea* (23.1%). Conversely, of the 11 wild cohorts tested three (27.3%) came back positive and these were all *L. raniformis.*

The other group tested for the presence of Bd were the alpine newts *Ichthyosaura alpestris.* This group of individuals were tested to confirm the prevalence of Bd in the population: they were tested individually using the standard RT-PCR. These animals were discovered in New Zealand in 2013 after having been illegally smuggled into the country and released. At the time of testing over 3000 individuals had been caught. A sample of 102 alpine newts were tested including five Juveniles. The newts were tested singularly for positive/negative result. The testing was done at Manaaki Whenua Landcare Research and the protocol was as above, with the exception being that the newts were swabbed then kept in separate containers with an individual identification number with the intention to isolate Bd zoospores from newts that were infected at a later date. Of the 102 Newts tested 52 tested positive for Bd and a further 4 where equivocal with only 2/3 (n=2) or 1/3 (n=2) replicates showing a positive result. The attempt to isolate Bd from the positive newts proved unsuccessful.

The *Litoria aurea* Samples Using Quantitative PCR Technique.

The second data set was of wild caught *L. aurea*. In this study the individuals had to be adult frogs and have a snout vent measurement greater than 35mm long. All individuals meeting these criteria were PIT tagged, swabbed, sexed, and morphological measurements were taken.

The data collected and used for the quantitative PCR part of the study allowed for a more depth analysis. By using quantitative PCR, the infection load of individuals could be assessed. Comparisons could then be made between sites and also between males and females, fertile males and gravid females among

Of the 603 individuals caught 440 were large enough to be PIT tagged and of those, recapture confirmed 440 of the PIT tags were working. Of the 441 recorded individuals with functional PIT tags 438 were swabbed for Bd using the Hyatt 2007 protocol as above. The two individual who were excluded from the study were from the third study site in Wahi. It was decided not to include this site because of the lack of taggable adults. The population consisted of juveniles that had recently completed metamorphosis and two adult gravid females, 79 juveniles were captured but because they could not be tagged it was unclear how many of these were recaptures.

For this study an individual was considered positive if the PCR result was greater than zero. There were several individuals that scored under one zoospore equivalent. These were included in the positive results, as although they fall outside the standard, they should still fall within a standard curve as it is reasonable to assume that the curve continues below one zoospore, as it would continue above the higher end of the standard curve. Over the two seasons there were 50 individuals that had a zoospore count under one: (n=18 season one, n= 32season two). If data is looked at by site, the Ocean Beach had 10 individuals with <1 zoospore and the monastery site 40 individuals <1 zoospore.

The number of positives for season one was 88 individuals out of 170 (51.8%), compared to 139 individuals out of 268 (51.9%) that tested positive in season two.

Table **5**.1 Zoospore count from Ocean Beach and Monastery site divided by season.

Season	Number Swabbed	Number Positive	Percentage	Zoospore Lower Limit	Zoospore Higher Limit
1	170	88	51.8	0	1940.518
2	268	139	51.9	0	7660.346

Table 5.2 Monastery site: broken down by season, zoospore count and percentage of positives.

Monastery Season	Number Swabbed	Number Positive	Number <1 Zoospore	Number >1 Zoospore	% Positive	Zoospore Higher Limit
1	65	38	9	29	58.46	358.555
2	42	28	1	27	66.67	3934.0951

Table 5..3 Ocean Beach site: broken down by season, zoospore count and percentage of positives.

Ocean Beach Season	Number Swabbed	Number Positive	Number <1 Zoospore	Number >1 Zoospore	% Positive	Zoospore Higher Limit
1	105	50	9	41	47.6	1940.5183
2	226	111	31	80	49.2	7660.3458

Table 5.4 Positive females from both sites' vs fertility.

Season	Gravid	Not Gravid	Total Females	Dark Nups	Light Nups	Clear Nups	Total Males	No Data
1	14	4	18	28	10	30	68	2
2	2	41	45	47	17	30	94	2

Table 5.5 Negative females from both sites' vs fertility

Season	Gravid	Not Gravid	Total Females	Dark Nups	Light Nups	Clear Nups	Total Males	No Data
1	20	3	23	49	1	4	44	5
2	1	49	54	68	4	3	75	4

Percentage of gravid females positive for Bd was 28% over both seasons. Separating seasons when looking at gravid females being positive season one 82.9% and season two 3.0%.

Site fertility and positive Bd infections

Table 5.6 Gravid females vs not gravid separated by site

Site	Gravid Pos	Not Gravid Pos	Gravid Neg	Not Gravid Neg	Total Females	% Gravid Pos	% Not Gravid Pos
Monastery	4	15	3	6	28	14.3	53.6
Ocean Beach	12	30	18	46	106	11.3	28.3

The odds of being gravid and positive = gravid positive/gravid negative

Monastery = 4/3=1.33, Ocean beach 12/18=0.67

Being not gravid positive/ not gravid and negative/ =

Monastery=15/6=2.5, Ocean beach =30/46= 0.65

Odds Ratio

Monastery= 1.33/2.5=0.53

Ocean Beach=0.67/0.65=1.03

The females at the Monastery site have approximately half the chance (odds ratio = 0.53) of being gravid when they are infected with Bd. Meanwhile, the females at the Ocean Beach site have the same chance (odds ratio = 1.03) of being gravid with a Bd infection.

Table5.7 Male fertility from each site and Bd infection.

Site	Dark Nups Pos	Dark Nups Neg	Clear Nups Pos	Clear Nups Neg	Total Males	% Dark Nups Pos	% Clear Nups Pos
Monastery	12	23	28	4	67	17.9	41.79
Ocean Beach	63	94	32	3	192	32.81	16.67

The males exhibiting dark nuptial pads were considered to be fertile. Any individual with pale nuptial pads was considered equivocal and excluded from the fertility data.

The odds of being fertile and positive = fertile positive/fertile negative

Monastery = 12/23=0.52, Ocean beach 63/94=0.67

Being not fertile positive/ not fertile and negative/ =

Monastery=28/4=7, Ocean beach =30/46=0.65

Odds Ratio

Monastery= 0.52/7=0.07

Ocean Beach=0.67/0.65=1.03

The males at the monastery were unlikely to be fertile and be positive with chytrid only a 0.07 odds ratio. Conversely the males at the Ocean Beach site had the same chance of being fertile with Bd as the females did being gravid with Bd; (odds ratio = 1.03).

Initially it was planned to do a third mark and recapture site for *L. aurea* as part of the larger Newcastle university study at the same site as the alpine newts were being captured. However, this did not prove feasible as the frogs were too small to be PIT tagged. Of the 82 captures over two nights, there were only two frogs, both gravid females that were big enough to be PIT tagged and each of these females was caught on consecutive nights. Therefore 78 captures were either Gosner stage 43 plus (Gosner, 1960) or newly transformed adults which could not be identified.

Discussion

The Bd fungus was present in three of the four different types of exotic amphibian populations in New Zealand; wild populations, the incursion population and the pet trade all had Bd positive individuals. Amphibians from the confinement facilities, Auckland Zoo and the University of Otago, all tested negative.

Amphibian chytrid fungus is present at a relatively high prevalence in wild *Litoria spp*. and *I. alpestris* throughout New Zealand. Approximately a half of all wild caught animals tested positive for Bd in the University of Newcastle study. The results for the Trade Me^{TM} and wild caught sample were lower, at

23.1% for the Trade Me^{TM} individuals and 27.3% wild caught being positive. The two samples are not comparable as one measured prevalence in a population while the Trade Me^{TM} and pet shop data recorded Bd presence over different populations and species. Furthermore, the Trade Me^{TM} sample was smaller.

Clearly, New Zealand populations of *Litoria* spp. are surviving with the presence of Bd in the population. It is unclear how a level of 50.0% prevalence is affecting these populations. There are several hypotheses that could be explored regarding the survival and or maintenance of these Bd infected populations. Increased immunity in the host species or reduced virulence of the pathogen (Altizer, Harvell, & Friedle, 2003). The infected amphibian populations may survive with a reduced population density (Briggs, Knapp, & Vredenburg, 2010; Briggs, Vredenburg, Knapp, & Rachowicz, 2005; Tobler, Borgula, & Schmidt, 2012). The lack of reservoir hosts in these sites may also increase survivorship of the *Litoria* spp. present: as it has been recorded that the presence of a reservoir host can increase mortality and reduce population size (L. Brannelly et al., 2018).

The eastern Waikato site where the *I. alpestris* were being eradicated was unusual in that the population of *L. aurea* at this site largely comprised of individuals that had recently completed metamorphosis. Of the ninety frogs captured there were only two adult females, and both were gravid. The rest of captures were of newly metamorphosed individuals that were too small to PITT tag. Because of this, the site was excluded from the larger *L. aurea* study. This site seemed to be struggling or in decline, as the only site that a reservoir species was present it was interesting to note the population dynamics with the near absence of adult frogs compared to the high number of juveniles. As *I. alpestris* predate on tadpoles: they are generalist predators and will eat tadpoles (B. D. Bell, 2016), it could be expected that this would cause a decline in their numbers. However, the reverse appeared to be the case with fewer adults and many juveniles observed at the site. This suggested Bd infection load may have been the contributing factor to the low number of adult frogs. There is evidence that as tadpoles' complete metamorphosis they are more susceptible to Bd infections. Bakar et al. found *L. aurea* frogs that had just completed metamorphosis, then exposed to Bd, had a much higher infection load and lower survivorship than adults and sub-adults that were exposed (Bakar et al., 2016). The survivorship of the metamorphs may have been affected by Bd infection at a vulnerable life stage. Conversely, if predation was the issue it would be expected that fewer juvenile individuals would be present as they would have been predated upon. This site could have provided insight into the dynamics of sympatric asymptomatic predator species on the survivorship of a vulnerable population. However, as the alpine newts where classed a risk to the endemic *Leiopelma archeyi* an eradication program was affected immediately (B. D. Bell, 2016).

The infection load of Bd has a positive correlation to mortality: the higher the infection loads the more likely mortality will be the outcome (Briggs et al., 2010; Jani & Briggs, 2014; Vredenburg, Knapp, Tunstall, & Briggs, 2010). Infection load is relative to the number of zoospores an individual is calculated to be infected with this can be determined through swabbing and quantitative PCR (Longo, Burrowes, & Joglar, 2010). Infection load is affected by the immune response of the amphibian which can be a result of many variables such as life stage (Bakar et al., 2016), skin microbes (Harris et al., 2009), and temperature (Sasha E. Greenspan et al., 2017) to name a few.

Previous research has found that increased salinity reduces Bd infection loads (Stockwell, Clulow, & Mahony, 2015) The difference in prevalence between the Ocean Beach site could be explained by the slight increase in the salinity of the Ocean Beach ponds. Although the spore count range was higher at Ocean Beach up to 7660.3 genomic equivalents, with an average count of 249.41 (331.77 excluding the individuals with <1 zoospore) genomic equivalents. Compared to the Monastery ponds up to 3934.095 genomic equivalents with an average of 205.08 (208.24 excluding the individuals with <1 zoospore) genomic equivalents. The only other measured difference was the water temperature between the ponds, Ocean Beach being cooler with temperature ranges between 10-17.5°C, while the Monastery Pond was between 19.2-24°C. It has been well documented that Bd does not survive higher temperatures (Longcore J.E. et al., 1999; Stevenson L.A. et al., 2013; Van Sluys M. et al., 2008). The Ocean Beach ponds although having slightly higher salinity were the ideal Bd growing temperature range of 10-17°C. The synergy between these two factors could be predicted to influence the incidence and or infection load of the frogs. However other factors such as the use of agrochemicals (Rohr et al., 2013), ultraviolet radiation exposure (Ortiz-Santaliestra, Fisher, Fernández-Beaskoetxea, Fernandez-Beneitez, & Bosch, 2011; Walker et al., 2010) and seasonality all influence Bd infection rate and host morbidity.

The effect of Bd infection has on fertility and reproduction has not been widely researched in amphibians however preliminary research suggests that amphibians do fit into the terminal investment hypothesis (L. A. Brannelly, Webb, Skerratt, & Berger, 2016). The terminal investment hypothesis proposes that energy invested into one last effort in breeding is more reproductively fit than investing in overcoming a disease and having future breeding opportunities (L. A. Brannelly et al., 2016; Duffield, Bowers, Sakaluk, & Sadd, 2017). In this study the results were equivocal between the two sites, while the monastery site suggested a decrease in both male and female fertility and reproductive output the Ocean Beach showed no difference. An explanation of this could be related to the higher zoospore count in the individuals at the Ocean Beach site (Ocean beach season 1 n=1940.5183, season 2 n= 7660.3458 vs Monastery season 0ne n = 358.555, Season 2 n =3934.0951). Looking at all the 13 individuals from both sites that had zoospore counts >1000 zoospore equivalents 10 out of the 13 were from Ocean Beach.

The incidence of Bd in *I. alpestris* was similar to the *L. aurea* populations 50.0% of animals testing clear positive and a further 3.8% being equivocal. Although this could be considered on the low side of actual prevalence as the standard PCR analysis was used and therefore only positives between the set standards for each test would be included. The site of the alpine newt incursion was interesting, with 82 captures of frogs, the majority of these could not be identified so could be recaptures; apart from two adult gravid females which where each captured twice. All the other frogs captured at the site where individuals that had recently completed metamorphosis.

Globally the anthropogenic movement of amphibian has been linked to the recent panzootic (Fisher & Garner, 2020; Schloegel et al., 2009). Unlike many countries the importation of animals via the pet trade into New Zealand is strictly controlled at the border, currently live amphibians are not imported for the pet trade. However, movement of existing animals is unregulated. With both wild stock and pet trade animals being infected with Bd the risk of a new strain evolving is a risk that should be mitigated. The hybridization (S. E. Greenspan et al., 2018) or recombination (Fisher & Garner, 2020) of a new more virulent strain would put our endangered endemic *Leiopelma* species at risk. Currently there are at least six species of amphibian in the pet trade that are capable of being infected with Bd fungus. These are all moved freely around the country from many different source's, hence, different strains of Bd may come into contact. It is unclear how many strains of Bd there are currently in the country. The first one, isolated by Stephanie Shaw was KVLe08SDS1, it was obtained from a *L. ewingii* individual found in Dunedin (S. D. Shaw, Bishop, Skerratt, Myhre, & Speare, 2014). I know of two that have been isolated by Russel Poulter at The University of Otago (RTP4 and RTP5), there is also RTP6 that is a recent isolate from a *L. ewingii* tadpole in Dunedin (Sumpter, Butler, & Poulter, 2018) all appear to be variants of the BdGPL line of Bd that recently came out of Asia via the amphibian trade (Fisher & Garner, 2020; Sumpter et al., 2018). With the potential of co-infections that could result in animals from different sources as in the pet trade, the risk of a recombination event is high. As (Fisher & Garner, 2020) describe co-infection can give rise to recombination events that can result in a more aggressive strain.

Recommendations

There should be a focus on mitigating the spread of chytridiomycosis via the pet trade. Regulation in this area could be as simple as reducing or minimising the distances amphibians can be moved. Reducing the anthropogenic movement of amphibians is important in mitigating disease transmission. Educating the public about it being illegal to release stock into New Zealand's waterways, currently most people are unaware of this law. A focus on getting this information out to schools would be one oof the most effective ways to educate the public as teachers have captive audiences and children then relay this information to parents. Furthermore, making information available regarding the best husbandry and hygiene practices when keeping amphibians as pets.

The more information and data that can be collected from wild populations will help in understanding population dynamics. This is particularly pertinent in eradication programmes. The eradication of the incursion species *I. alpestris* was an opportunity missed. In the rush to eradicate the newt population valuable opportunities were such as population demographics, age, sex, and density of individuals were missed. Collecting data from the newt population had the potential to establish how long the newt population had been naturalised in the location. Furthermore, the site could have provided valuable insight on the effects an invasive species has on existing invasive populations: *L. aurea* in this instance. Closer working relationships between wildlife management authorities and researchers need to be adopted so important opportunities that will benefit both sides are utilised providing a better understanding of wildlife dynamics.

Publishing results and data are imperative for the progress of research and to allow future researchers to build on previous research and knowledge. To assess the risk of Bd to New Zealand's amphibians, being able to access literature on the strains of Bd that have already been identified would provide valuable insight into the chance of a more virulent strain developing via a recombination event. For this to be achieved testing of all alien amphibians crossing the border should be considered and where applicable isolating the strain of Bd.

Summary

Batrachochytrium dendrobatids is found in wild populations of amphibians in New Zealand. With the lack of regulation regarding the movement, keeping and selling these amphibians it would be expected that the anthropogenic movement of infected animals and thereby Bd throughout the country will continue. This poses a risk in the development of a new more virulent strain of Bd that could threaten the endangered *Leiopelma* species. Restricting the human mediated movement of amphibians could help mitigate the spread of Bd.

Litoria spp. are surviving as wild populations with Bd prevalence's of around 50.0%. It is unclear how they are maintaining populations with this level of infection or if indeed the population sizes are stable. Whether they are developing resistance to the strain of Bd in the population or have an increased reproduction rate when infected with high levels of Bd should be investigated further.

Preliminary evidence suggests that when there is a reservoir host such as *I. alpestris* that is also a predator of the susceptible species like *L. aurea* population decline will be extreme and extirpation likely.

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6 Conclusions

The primary finding of this research was identifying that the introduction and spread of new pathogens poses a greater risk to New Zealand's endemic *Leiopelma* spp. rather than the establishment of an invasive species of amphibian. This is primarily because of their biphasic life cycle and specific survival requirements. Not only are amphibians susceptible to dehydration and they most require water to breed, but unlike reptiles, they are rarely viviparous. Only two species of Salamandra (*S. salamandra* and *S. algira*), and of the anurans the genus of toads Nectophrynoides are viviparous. So, for a population to become established there would need to be several sexually mature adults arriving together in the breeding season and having access to a suitable body of water. This would be more likely to occur if the animals were smuggled and later released, as in the case of the European alpine newts. In contrast, the risk of a pathogen becoming established would be greater. It could take a single infected individual that had not been handled and disposed of following strict disease prevention protocols. This includes all the equipment and substrate that the animal came into contact with. Alternatively, the accidental escape of a single infected individual could allow the establishment of a novel pathogen into naive habitats and populations.

This study of the prevalence of amphibian chytrid in different amphibian populations in New Zealand, and possible disease pathways, has re-confirmed the presence of Bd in populations of *Litoria* spp., it has also uncovered some key risk areas of transmission.

These include:

- The movement of frogs and tadpoles via the pet trade.
- The interspecies contact between different subclasses of amphibians through the pet trade.
- The release of pet frogs into the wild.
- Lack of hygiene guidelines in both the pet trade and with authorities dealing with amphibian incursions.

In the two populations of wild *Litoria aurea* in this study, the prevalence of Bd infection was around 50.0%. It is unclear how they are maintaining an infection rate at this level, and how long these populations can survive with such a high disease prevalence. Several hypotheses might explain my findings:

- The frogs are developing resistance to Bd.
- The frogs have increased reproductive output and, as a result, population levels are maintained.

- *Batrachochytrium dendrobatidis* is becoming less virulent, in at least some strains in New Zealand.
- A combination of all the above or other biotic or abiotic factors e.g., the absence of asymptomatic reservoir species.

Further research in these areas, especially longitudinal studies that could reveal how populations change over time, could help answer these questions. Future research should include not just laboratory studies but wild populations, as the dynamics of wild populations of infected amphibians and disease responses often differ when compared to controlled laboratory experiments.

To expand our understanding of the prevalence Bd in New Zealand future studies should focus on areas with poor data, such as the amphibian populations in the lower North Island and the South Island. A better understanding of the prevalence rates in the two other *Litoria* spp. *L. raniformis* and *L. ewingi* in these regions would be valuable.

The risk to New Zealand's four *Leiopelma* species still needs further investigation. Although the endemic *Leiopelma* frogs do not generally live sympatrically with the introduced *Litoria* frogs it has been recorded that at least in the Whareorino populations of the two *Leiopelma* species *L. archeyi* and *L. hochstetteri* the introduced *L. aurea* was not only living sympatrically but there was evidence that *L. aurea* predated on *Leiopelma*. Even if the *Litoria* frogs do not live sympatrically they are known to travel large distances during their breeding season. An animal infected with Bd passing through previously uninfected areas that are damp or coming into contact with water bodies risks spreading Bd zoospores, which can survive for many weeks without a host.

Although it has been found that adult *L. archeyi* have self-cured from Bd exposure, all the animals were adults. It has been noted that resistance to Bd changes over life history with new metamorphs having higher mortality (Bakar et al., 2016). The population crash recorded in 1996-2001 also documented the snout-vent length (svl) of captured individuals. It is interesting to note that svl decreased after the population crash suggesting fewer younger individuals were captured. The breeding of *L. archeyi* with the male brooding the metamorphs on his back would expose the young frogs to Bd zoospores at a time when the amphibian immune system is being remodelled (Louise A. Rollins-Smith, 1998; Louise A Rollins-Smith, 2001). If exposed to Bd at this life stage there is a high risk of mortality (Fernández-Loras, Fernández-Beaskoetxea, Arriero, Fisher, & Bosch, 2017).

The anthropogenic movement of amphibians nationally is significant; my study revealed over 10,000 amphibians a year are sold via pet shops and Trade Me^{TM}. With the vast majority of these individuals being collected from the wild, it can be assumed that the anthropogenic distribution of chytridiomycosis throughout New Zealand was/is not only widespread but extremely rapid. The combination of pet shops and Trade Me^{TM} provides pathways for highly effective human-assisted dispersal of introduced amphibians, ensuring the proliferation of this disease. These two trading methods not only aid disease pathways but also intensify them: pet shops bring in many species from many sources and distribute them to single locations (e.g., local homes). Conversely, 'trade me' sellers collect frogs from local areas and distribute them nationally. By restricting the movement of amphibians by humans, the current disease pathways could be slowed considerably.

Human-animal contact has health risks, New Zealand, as a remote island nation, has been historically, relatively free from many zoonoses (Crump, Murdoch, & Baker, 2001). Probably the most well-known zoonoses in New Zealand are tuberculosis, campylobacteriosis, salmonellosis, leptospirosis and toxoplasmosis, and historically hydatids. Less serious zoonoses included ringworm, ticks, and fleas. In their 1987 publication 'Zoonoses in New Zealand' Blackmore and Humble list 28 zoonotic diseases present in New Zealand. Since then, several like hydatids and brucellosis have been eradicated and many others like tuberculosis and leptospirosis have deceased in their incidence (Wilks & Humble, 1998). The majority of the diseases listed are from domesticated animals, however, any interaction between humans and animal's risks disease transmission including diseases from wildlife. A relatively recent example was the discovery of the Malaysian trumpet snail (*Melanoides tuberculata*) thought to have been introduced accidentally via the pet trade (Duggan, 2002). The snail is an intermediate host for several trematodes that are known human parasites overseas (Derraik, 2008). *Melanoides tuberculate*, like *Litoria* spp., have been sold widely on the online auction site Trade Me, which may account for its spread to other locations. As human-wildlife interactions increase the need to mitigate zoonoses will become increasingly important. The pet trade could be considered a high-risk area, not only because of the increased interaction between humans and animals but from the bottleneck effect from bringing many different species together that are then distributed widely out into the New Zealand environment. The release of pets into the wild intensifies the risk of disease spread, this is an area that could be targeted to help mitigate the risk of transmission. The increase availability of educational material about amphibians present in New Zealand and the inclusion of this information into school curriculum are two ways to achieve this.

There were several areas that could be addressed to help mitigate exotic amphibian introductions and their associated diseases entering New Zealand. These include identifying smuggled amphibian pathways. Of the three instances of amphibians being smuggled into New Zealand the entry pathway has not been established or reported (at least in the literature). A New Zealand Geographic article about smuggled reptiles revealed the postal system as one entry point for exotic reptiles to enter New Zealand (https://www.nzgeo.com/stories/the-reptile-smugglers/). Furthermore, they cite events where smugglers have been intercepted trying to leave the country with endemic species. Australian border data reported the animals being smuggled would be carried on the passenger or in their luggage (Henderson & Bomford, 2011). The likelihood of these two pathways being the main entry points for exotic species entering New Zealand is high. Live animals need to be transported rapidly if they are going to survive, although many of them perish (Morgan, 2015).

Borders staff and staff involved with amphibian incursions should treat all amphibians as though they are diseased, including the medium in which they are transported. A good working relationship wildlife management and researchers should be encouraged and nurtured so opportunities are not missed that would benefit both parties. My experience and personal observations were that researchers were not utilised early enough in Alpine newt incursion and opportunities to gain valuable knowledge about disease dynamics and the naturalisation of an invasive species were missed.

Routine testing of intercepted amphibians for the main known diseases like Chytridiomycosis and Ranavirus should be mandatory. The current method of storing the entire specimen in 70.0% ethanol could be improved to increase disease detection if parts of each specimen were also stored in formalin and frozen. Finally, ongoing disease testing for Bd and subsequent analysis of the strain is important, as any future strains that enter the country risk combination with the current strain resulting in a more virulent strain.

Pet shops and Trade Me^{TM} sellers pose contrasting disease risk factors in the amphibian pet trade. While any movement of animals increases the of disease transmission into previously disease-free areas, by reducing the distance animals are moved will help slow the spread of the pathogen.

Comparing the movement of animals between Pet shops and Trade Me^{TM} sellers highlighted the different modalities of risk. Trade Me^{TM} sellers collected tadpoles from their local environment and posted them nationwide. They mostly only traded in a single species. Conversely pet shops obtained their stock from multiple sources where suppliers were not local. Their clientele would most likely be locals. Pet shops also traded in many species. Between these two methods of trading, disease pathways are intensified: pet shops bring in many species from many sources and distribute them to single locations (e.g., local homes). Conversely, 'Trade Me^{TM}' sellers collect frogs from local areas and distribute the nationally (many locations).

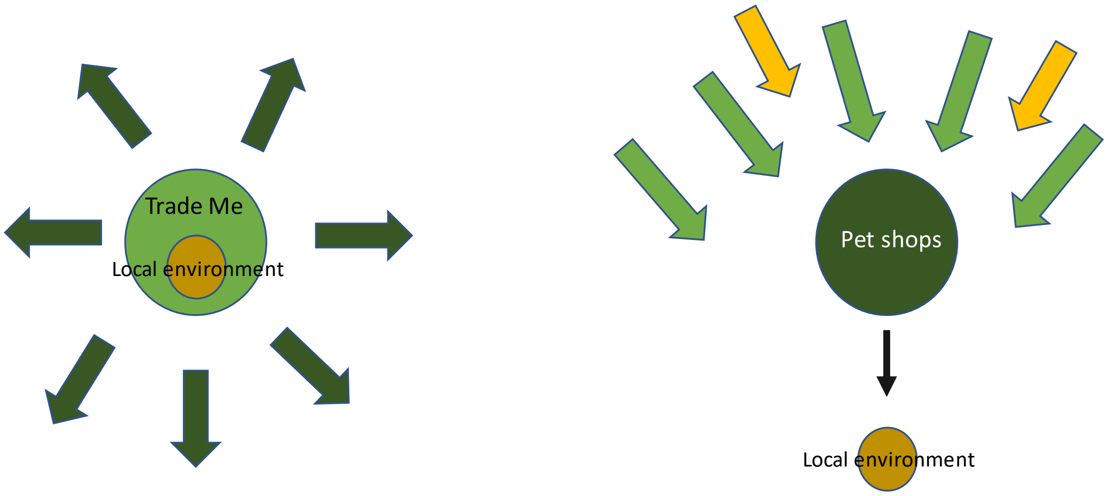

Figure 6. 1 Disease transmission pathways Trade Me versus pet shops.

While there are many opportunities for disease transmission in amphibians in the pet trade, in New Zealand the risks related to this should be balanced with the enjoyment and education that amphibians provide. Amphibian ownership of *Litoria* spp. in New Zealand should be encouraged with the support and distribution of educational material. This will support the future vested interest in amphibians and aid in their survival.

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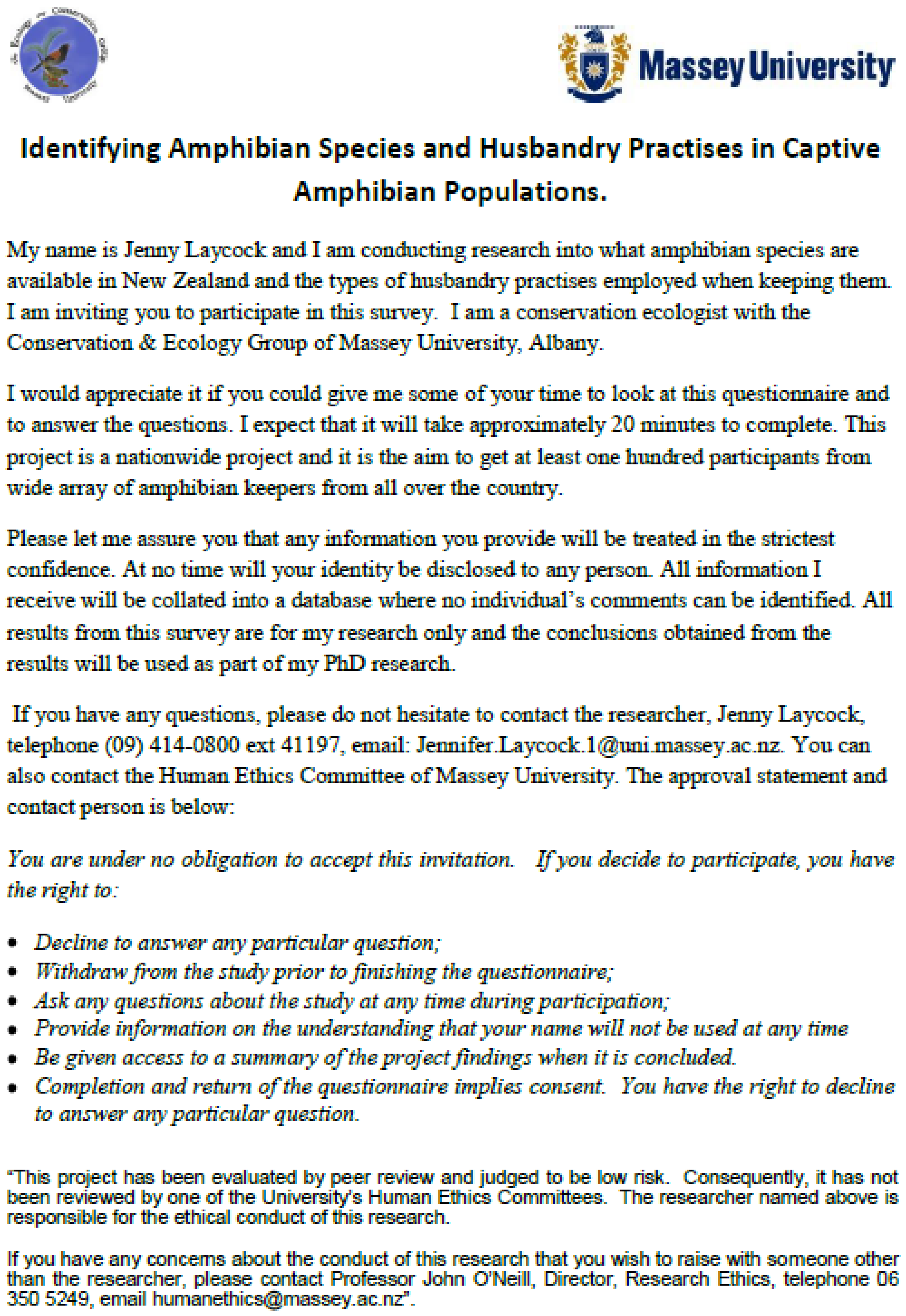
Appendix

Information Sheet for the Amphibian Survey



Identifying Amphibian Species and Husbandry Practises in Captive Amphibian Populations.

My name is Jenny Laycock and I am conducting research into what amphibian species are available in New Zealand and the types of husbandry practises employed when keeping them. I am inviting you to participate in this survey. I am a conservation ecologist with the Conservation & Ecology Group of Massey University, Albany.

I would appreciate it if you could give me some of your time to look at this questionnaire and to answer the questions. I expect that it will take approximately 20 minutes to complete. This project is a nationwide project and it is the aim to get at least one hundred participants from wide array of amphibian keepers from all over the country.

Please let me assure you that any information you provide will be treated in the strictest confidence. At no time will your identity be disclosed to any person. All information I receive will be collated into a database where no individual's comments can be identified. All results from this survey are for my research only and the conclusions obtained from the results will be used as part of my PhD research.

If you have any questions, please do not hesitate to contact the researcher, Jenny Laycock, telephone (09) 414-0800 ext 41197, email: Jennifer.Laycock.1@uni.massey.ac.nz. You can also contact the Human Ethics Committee of Massey University. The approval statement and contact person is below:

You are under no obligation to accept this invitation. If you decide to participate, you have the right to:

- *Decline to answer any particular question;*
- *Withdraw from the study prior to finishing the questionnaire;*
- *Ask any questions about the study at any time during participation;*
- *Provide information on the understanding that your name will not be used at any time*
- *Be given access to a summary of the project findings when it is concluded.*
- *Completion and return of the questionnaire implies consent. You have the right to decline to answer any particular question.*

"This project has been evaluated by peer review and judged to be low risk. Consequently, it has not been reviewed by one of the University's Human Ethics Committees. The researcher named above is responsible for the ethical conduct of this research.

If you have any concerns about the conduct of this research that you wish to raise with someone other than the researcher, please contact Professor John O'Neill, Director, Research Ethics, telephone 06 350 5249, email humanethics@massey.ac.nz".

Participant Consent Form Amphibian Survey

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Identifying Amphibian Species and Husbandry Practices in Captive Amphibian Populations.

PARTICIPANT CONSENT FORM - INDIVIDUAL

I have read the Information Sheet and have had the details of the study explained to me. My questions have been answered to my satisfaction, and I understand that I may ask further questions at any time.

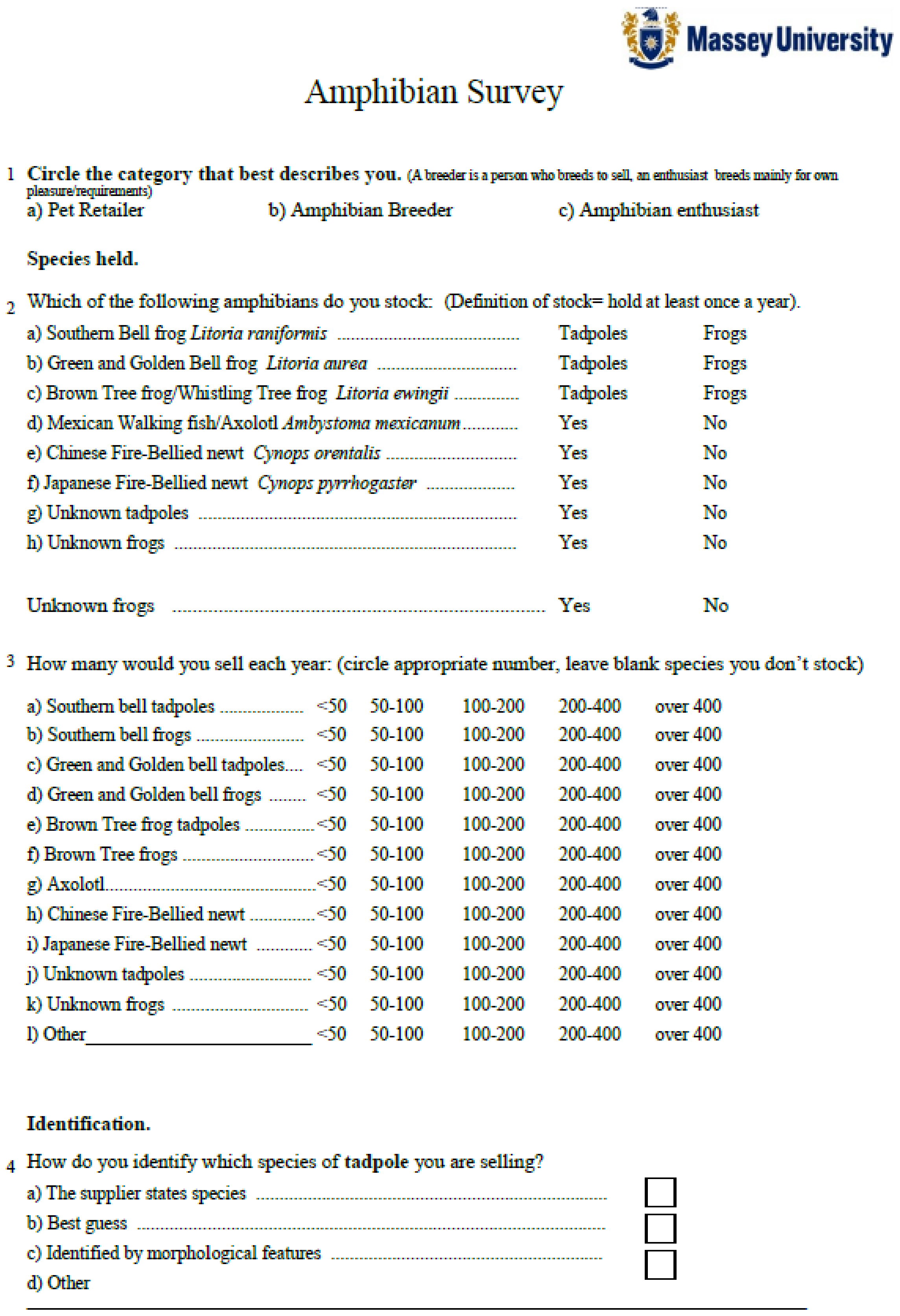
I agree to participate in this study under the conditions set out in the Information Sheet.

Signature: -- **Date:** ------------------

Full Name - printed ---

Format for Participant Consent Form (2009) Page 1 of 1

Amphibian Survey



Massey University

Amphibian Survey

1 **Circle the category that best describes you.** (A breeder is a person who breeds to sell, an enthusiast breeds mainly for own pleasure/requirements)

a) Pet Retailer　　b) Amphibian Breeder　　c) Amphibian enthusiast

Species held.

2 Which of the following amphibians do you stock: (Definition of stock= hold at least once a year).

a) Southern Bell frog *Litoria raniformis*	Tadpoles	Frogs
b) Green and Golden Bell frog *Litoria aurea*	Tadpoles	Frogs
c) Brown Tree frog/Whistling Tree frog *Litoria ewingii*	Tadpoles	Frogs
d) Mexican Walking fish/Axolotl *Ambystoma mexicanum*	Yes	No
e) Chinese Fire-Bellied newt *Cynops orentalis*	Yes	No
f) Japanese Fire-Bellied newt *Cynops pyrrhogaster*	Yes	No
g) Unknown tadpoles	Yes	No
h) Unknown frogs	Yes	No
Unknown frogs	Yes	No

3 How many would you sell each year: (circle appropriate number, leave blank species you don't stock)

a) Southern bell tadpoles	<50	50-100	100-200	200-400	over 400
b) Southern bell frogs	<50	50-100	100-200	200-400	over 400
c) Green and Golden bell tadpoles....	<50	50-100	100-200	200-400	over 400
d) Green and Golden bell frogs	<50	50-100	100-200	200-400	over 400
e) Brown Tree frog tadpoles	<50	50-100	100-200	200-400	over 400
f) Brown Tree frogs	<50	50-100	100-200	200-400	over 400
g) Axolotl..	<50	50-100	100-200	200-400	over 400
h) Chinese Fire-Bellied newt	<50	50-100	100-200	200-400	over 400
i) Japanese Fire-Bellied newt	<50	50-100	100-200	200-400	over 400
j) Unknown tadpoles	<50	50-100	100-200	200-400	over 400
k) Unknown frogs	<50	50-100	100-200	200-400	over 400
l) Other______________________	<50	50-100	100-200	200-400	over 400

Identification.

4 How do you identify which species of tadpole you are selling?

a) The supplier states species .... ☐

b) Best guess .... ☐

c) Identified by morphological features .... ☐

d) Other ____

5 Do you ever confirm the species of tadpole you are selling? (Circle appropriate number)

I Always confirm the species **1** **2** **3** **4** **5** I Never confirm the species

Records.

6 Do you keep records of where species are obtained from and who they are obtained from?

(NN= Name and number FD= Full details of supplier FDSL= Full details and source/ location of stock).

a) Southern Bell frogs	No	NN	FD	FDSL	N/A
b) Southern Bell tadpoles..................	No	NN	FD	FDSL	N/A
c) Green and Golden Bell frogs	No	NN	FD	FDSL	N/A
d) Green and Golden Bell tadpoles ...	No	NN	FD	FDSL	N/A
e) Brown Tree frog............................	No	NN	FD	FDSL	N/A
f) Brown Tree frog tadpoles...............	No	NN	FD	FDSL	N/A
g) Axolotl	No	NN	FD	FDSL	N/A
h) Chinese Fire-Bellied newt	No	NN	FD	FDSL	N/A
i) Japanese Fire-Bellied newt	No	NN	FD	FDSL	N/A
j) Unknown tadpoles	No	NN	FD	FDSL	N/A
k) Unknown frogs	No	NN	FD	FDSL	N/A
l) Other______________________	No	NN	FD	FDSL	

Husbandry.

7 When you get new stock of a particular species, do you: (tick as many as appropriate)

a) Place in aquarium with existing stock.. ☐
b) Quarantine then place in aquarium with existing stock .... ☐
c) Place in a new aquarium .... ☐
d) Quarantine then place in new aquarium .... ☐
e) Place in a sterilised aquarium .... ☐
f) Quarantine then place in new sterilised aquarium .... ☐
g) Other____

____

____

8 **Filtration.**

How are your display tanks filtered? (Circle appropriate answer)

a) Individually b) Small groups c) One system for all tanks

d) Other____

9 If tanks are filtered in groups, how are the groups organised? (Circle answer)

a) By position b) Size of Tank c) What species it holds

d) Other____

Waste water disposal.

10 How do you dispose of waste water?

a) Put straight into the drain .... ☐

b) Filtered and then put in drain.. ☐

c) Sterilised and then put into drain.. ☐

d) Other ____

Purchasing Amphibian Stock.

11 Rank in the order of priority '1' being of most concern '5' being of least concern:

When purchasing amphibian stock I:

a) Choose suppliers whose stock is located nearest to our premises .... ☐

b) Choose suppliers who provide the healthiest stock .... ☐

c) Choose suppliers who give me the best deal .... ☐

d) Use the same supplier I always have .... ☐

e) Choose a supplier who can provide all the species I require .... ☐

Equipment, Buckets, Nets, Gloves, etc.

12 How is equipment assigned?

a) Each tank has its own designated bucket, net and equipment........................ ☐

b) Each species has its own designated bucket, net and equipment........................ ☐

c) We do not designate equipment........................ ☐

d) Other ____

13 How often are equipment, buckets, nets, gloves cleaned? (Circle answer)

a) Daily b) Weekly c) Monthly d) Whenever it is dirty

e) Other____

14 How is the equipment cleaned?

a) Rinsed under water until debris is not visible .... ☐
b) Rinsed and left to dry completely in the sun .... ☐
c) Washed with hot water and soap .... ☐
d) Sterilised using a disinfectant. (Which brand of disinfectant?) ______________ ☐
e) Sterilised using UV.. ☐
f) Sterilised by boiling for 5 or more minutes.. ☐
g) Other____

Mortality.

15 Do you keep a record of mortalities? **Yes** **No**

If yes, what information is included? (Circle as many as appropriate).

a) Species b) Date c) Cause of death d) Post Mortem results e) Number of dead

d) Other ____

Disposal.

16 How do you dispose of dead amphibians?

a) Put in rubbish bin b) Biohazard waste c) Incinerate d) Flush down drain

e) Other____

Advice at purchase.

17 Do you give husbandry advice at time of purchase? (Circle appropriate number)

a) Verbal .... Never 1 2 3 4 5 Always
b) Written .... Never 1 2 3 4 5 Always

18 Is the husbandry advice provided for all species? **Yes** **No**

If no, which species do you offer it for: ____

19 Do you offer after purchase support? (Circle appropriate number)

Never 1 2 3 4 5 Always

What support do you offer? Written Verbal

Other____

20 Is the after purchase support advice provided for all species? **Yes** **No**

If no, which species do you offer it for: ______________________________________

Amphibian Knowledge.

21 How would you rate your knowledge of amphibians on a scale of 1 to 5. (Circle appropriate number)

Limited 1 2 3 4 5 **Expert**

22 How many species of Frog are in New Zealand (circle answer)

1 2 3 4 5 6 7 8 9 Don't know

23 **Frogs and the law.**

Below is a set of statements, circle what you believe is the correct answer.

a) New Zealand has no native species of frog. .... True False

b) All species of frog are allowed to be kept in New Zealand without a permit............. True False

c) Anyone can collect frogs and tadpoles from the wild. .... True False

d) You can release frogs.. True False

e) You can release tadpoles. .... True False

f) It is illegal to release frogs or tadpoles in New Zealand. .... True False

g) You are allowed to release frogs as long as it is back to the same locale................. True False

h) Some of the frogs that you see and hear in ponds around New Zealand are native.... True False

i) You need permission to collect some species of frogs. .... True False

j) Frogs are not protected by any laws in NZ.. True False

k) All frogs in New Zealand are native .... True False

Thank you for taking part in this survey