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Screening for *Batrachochytrium dendrobatidis* in New Zealand native frogs: 20 years onAdria Rae Abigail R. Eda^{1,2,3*} , Phillip J. Bishop^{1†} , Joseph T. Altobelli¹ , Stephanie S. Godfrey¹  and Jo-Ann L. Stanton² ¹Department of Zoology, University of Otago, Dunedin 9054, New Zealand²Department of Anatomy, University of Otago, Dunedin 9054, New Zealand³School of Biological Sciences, Victoria University of Wellington, Wellington 6012, New Zealand*Author for correspondence (Email: adriaeda05@gmail.com)

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Abstract: A chytrid fungus (*Batrachochytrium dendrobatidis*; Bd) has been a cause of amphibian declines worldwide. *Batrachochytrium dendrobatidis* was first detected in New Zealand on an introduced frog species in 1999 and two years later was associated with morbidity in *Leiopelma archeyi*, one of the three native New Zealand frog species. In this study, we aimed to document the prevalence of Bd in native frog species in New Zealand from 2014–2021. Skin swabs were collected from six sites in New Zealand: Maud Island/Te Pākeka, Zealandia Ecosanctuary, Auckland Zoo, and University of Otago between 2019–2021, and Whareorino Forest and Pukeokahu between 2014–2020. Swabs were analysed using qPCR to detect Bd from skin swabs. A total of 324 frogs from the six sampling sites were screened for Bd presence. Four percent of the *L. hamiltoni* and eight percent of the *L. archeyi* were Bd-positive, and all *L. hochstetteri* were Bd-negative. The detection of Bd in *Leiopelma* species confirmed that Bd persists in New Zealand endemic frogs at a low prevalence and intensity.

Keywords: amphibian, chytrid fungus, chytridiomycosis, disease prevalence, *Leiopelma*

Introduction

Chytridiomycosis is an infectious disease that has led to significant population declines and extinctions of amphibians worldwide (Skerratt et al. 2007; Pereira et al. 2013; Koo et al. 2021). The disease is caused by a fungal pathogen *Batrachochytrium dendrobatidis* (Bd) Longcore et al. 1999) and is currently considered the most significant threat to amphibians (Drew et al. 2006; Xie et al. 2016; Peralta-Garcia et al. 2018). However, in New Zealand, though still to be fully ascertained, Bd may have a more minor impact on native frog populations relative to other threats such as habitat loss and mammalian predators (Bishop et al. 2013).

Batrachochytrium dendrobatidis was first detected in New Zealand in 1999 after the discovery of sick Southern bell frogs (*Ranoidea raniformis*, an introduced species) in Christchurch (Waldman et al. 2001). In 2001, the death of a native frog (*Leiopelma archeyi*) on the Coromandel Peninsula was attributed to chytridiomycosis, but not definitively (Bell et al. 2004). Concern about the impacts of Bd on native frogs grew when a population of *L. archeyi* in the Coromandel region crashed between 1996–2001 (Bell et al. 2004). The discovery of chytridiomycosis in New Zealand caused great alarm, as frogs in the genus *Leiopelma* represent a primitive amphibian lineage (Roelants et al. 2005). The genotype of Bd discovered in New Zealand belong to the global panzootic lineage (BdGPL;

Sumpter et al. 2018), suggesting an overseas origin.

In 2006, Bd was also found in the *L. archeyi* population in Whareorino Forest (Bishop et al. 2009), so frogs were translocated to Pureora Forest, a location with suitable habitat for *L. archeyi* but with no documented evidence of Bd (Bishop et al. 2009). Three years after the translocation, Bd prevalence had increased in the Whareorino population but did not cause a further population decline in contrast to the Coromandel's *L. archeyi* population which continued to decline (Shaw et al. 2008; Shaw et al. 2010). Despite the emergence of Bd in *L. archeyi*, Bd has not been detected in the two other native frog species *L. hochstetteri* and *L. hamiltoni*. Bd has never been detected in *Leiopelma hochstetteri* (Moreno et al. 2011) the only semi-aquatic species that lives in sympatry with *L. archeyi* (Bell 1994) despite Bd being aquatic by nature and posing more of a threat to species that breed in aquatic environments (Kriger & Hero 2007). Bd has been detected in *R. aurea* and *Litoria ewingii*; both Australian frogs that have naturalised in New Zealand (Shaw et al. 2008; Moreno et al. 2011).

Melzer and Bishop (2010) explored antimicrobial peptides present on the skin of different native and introduced species. They showed that *L. archeyi* and *Li. ewingii* produced relatively high concentrations of antimicrobial peptides capable of inhibiting Bd *in vitro*. *Leiopelma hochstetteri* and *L. hamiltoni* both produced lower concentrations of protective peptides, but inhibition was strong. Shaw et al. (2014) conducted a

baseline inventory of the skin bacteria from *L. archeyi* and *L. hochstetteri* located in Whareorino forest and the Coromandel Peninsula to assess if these microorganisms could prevent chytridiomycosis. Challenge assays against Bd revealed that species of *Pseudomonas* and *Flavobacterium* were among the isolates inhibitory to Bd. However, further investigation is needed to fully understand the role of cutaneous microbes and immunity to chytridiomycosis (Shaw et al. 2014).

The resistance to Bd recorded in frogs from the genus *Leiopelma* suggests that chytridiomycosis may not pose a risk to leiopelmatid frogs (Bishop et al. 2009; Shaw et al. 2010; Ohmer et al. 2013). Despite the presence of Bd in at least two native frog species, and its presumed association with declines in *L. archeyi*, the current prevalence of Bd in wild and captive amphibian populations is unknown. To assess the current prevalence of Bd in New Zealand, we surveyed for the presence of Bd on the endemic frog species, *L. hamiltoni*, *L. archeyi* and *L. hochstetteri*, at both wild and captive/managed locations across New Zealand between 2014–2021 using a Taqman assay described by Boyle et al. (2004) and Hyatt et al. (2007). Bell et al. (2004) found that the mean condition index of *L. archeyi* was significantly greater after the earlier population decline that was thought to be associated to chytridiomycosis. To assess the relationships between Bd presence and body condition in *Leiopelma* species, we calculated the body condition of each frog and compared the body condition index between Bd-positive and Bd-negative animals. Lastly, in Whareorino Forest, where frogs were screened for Bd presence between 2014–2020, we estimated the Bd prevalence in each year to understand if there was a temporal trend in the prevalence of Bd in *L. archeyi*. This work provides insights into the current prevalence of Bd in New Zealand native frogs and supports the need for continued monitoring in *Leiopelma* populations across the country.

Methods

Sample collection

Frogs were sampled from six locations in New Zealand between October 2019 to February 2021 (Table 1). Skin swabs were collected from *L. archeyi* ($n = 248$) in two wild populations

(Whareorino and Pukeokahu) and one captive population (Auckland Zoo). Skin swabs were collected from *L. hamiltoni* ($n = 72$) in one wild population (Maud Island) and two captive populations (University of Otago and Zealandia Ecosanctuary). Skin swabs were collected from *L. hochstetteri* ($n = 14$) in a captive population only (University of Otago). Skin swabs from *L. archeyi* collected from Whareorino between 2014–2020 and from Pukeokahu in 2019 were provided by the Department of Conservation.

Frogs were captured by a gloved hand and placed individually in sterile 250 mL screw-capped pottles. Individual frogs were washed with 50 mL sterile Still™ spring water to remove soil debris and transient microorganisms. Each frog was then swabbed using a sterile medical dry swab (Thermo Fisher Scientific catalogue no. MWE100-100) on its ventral surface (Kriger et al. 2006). Swabs were stored at -20°C until DNA extraction was performed. Frogs were visually assessed for chytridiomycosis symptoms such as skin sloughing, lethargy, and redness of the pelvic patch. Since there are minimal morphological differences between male and female leiopelmatid frogs (Germano et al. 2011), captive frogs were the only reliably sex-identified individuals, where hormones in urine were used to identify sex. The snout-to-vent length (SVL, mm) and weight (g) of each frog was measured to calculate the body condition of the frogs at the time of sampling. Body condition was calculated as scaled mass index (\hat{M}_i) following the formula described by Peig & Green (2009):

$$\hat{M}_i = M_i \left[\frac{L_0}{L_i} \right]^{b_{SMA}} \quad (1)$$

where M_i is the body mass of individual i , L_i is the snout-vent length (SVL) of individual i ; b_{SMA} is the scaling exponent estimated by the standardised major axis (SMA) regression of M on L ; L_0 is the mean SVL of the population; and M_i is the predicted body mass for individual i when the SVL is standardised to L_0 .

Frogs were classified according to the type of population management they experienced, which dictated the environmental conditions. Wild population refers to locations where frogs are in their natural habitat with no direct human intervention (Whareorino Forest and Maud Island). Captive population refers to locations where frogs are monitored inside

Table 1. Number and species of leiopelmatid frogs sampled in different locations, with the sampling date and temperature (if known) at the time frogs were swabbed.

Frog Species	Location	Sampling Date	Average Temperature ($^{\circ}\text{C}$)	Number of Individuals
Wild population				
<i>Leiopelma archeyi</i>	Whareorino Forest	14 October 2019	8.5	39
<i>L. hamiltoni</i> ^a	Maud Island	9–11 October 2020	12.4	18
<i>L. archeyi</i> *	Whareorino Forest	2014–2020	10.6	158
<i>L. archeyi</i> *	Pukeokahu	14 November 2019	9.5	17
Captive indoor population				
<i>L. archeyi</i>	Auckland Zoo	17 December 2019	18.0	13
<i>L. hamiltoni</i> ^a	University of Otago	21–22 May 2020	15.0	23
<i>L. hochstetteri</i>	University of Otago	24 June 2020	16.0	14
Captive outdoor population				
<i>L. archeyi</i>	Auckland Zoo	17 December 2019	16.0	11
<i>L. hamiltoni</i> ^a	Zealandia Ecosanctuary	23 February 2021	18.4	31

^aformerly classified as *L. pakeka*

*Frog swabs were collected and provided by the Department of Conservation

a controlled environment. These frogs are housed inside tanks and fed on a schedule. Here, the temperature, humidity, and light exposure are set to mimic the frog's natural ecosystem (University of Otago and Auckland Zoo). Captive indoor populations refer to frogs housed in indoor enclosures, while captive outdoor populations refer to frogs housed in outdoor enclosures. These frogs are monitored with occasional human intervention and exposed to natural environmental conditions such as temperature, humidity, and sunlight (Zealandia Ecosanctuary and Auckland Zoo).

Extraction of DNA from swabs

DNA was extracted from each swab using 50 μL PrepMan™ Ultra Sample Preparation Reagent (Applied Biosystems, USA) following Boyle et al. (2004) with additional homogenisation process as described by Hyatt et al. (2007). Twenty microliters of the supernatant were transferred to a new sterile microcentrifuge tube and stored at -20°C . DNA samples were diluted 1:2 with tris-ethylenediamine tetraacetic acid (TE) buffer (pH 8) prior to qPCR assay. Dilution reduced possible PCR inhibitors that may be present in PrepMan extracts.

Preparation of Bd standards using gBlock gene Fragment and known Bd zoospore concentration

We followed the protocol as described by Rebollar et al. (2017) to prepare the Bd standards using gBlock gene fragment. A synthetic double-stranded gBlock (Integrated DNA Technologies, Singapore) gene fragment was used to estimate the number of Bd genomes extracted from individual swabs. A 206 bp gBlock was designed based on the partial sequence of Bd described by Boyle et al. (2004) to target the internal transcribed spacer (ITS) 1 and 5.8S ribosomal RNA gene based on GenBank accession number AY598034.1 (Appendix S1 in Supplementary Material). The gBlock was suspended using TE buffer following the manufacturer's instructions. A 2 ng μL^{-1} concentrated stock solution was prepared and stored at 9.48×10^{10} copies μL^{-1} . Bd gBlock standards were prepared by diluting the 9.48×10^{10} DNA copies/ μL concentrated stock solution to working solutions of 1×10^0 to 1×10^3 DNA copies μL^{-1} .

Bd zoospores were collected from a week-old Bd 1% tryptone agar plate culture. Spore concentration was determined using a haemocytometer. The spore suspension was diluted to 1×10^7 spore mL^{-1} . DNA was extracted from Bd zoospores following Boyle et al. (2004). The extracted Bd DNA, with an assumption of 100% extraction efficiency, has a concentration of 1×10^7 Bd spores in 150 μL which is equivalent to 6.7×10^7 spores mL^{-1} . Bd standards were prepared by diluting DNA to 1000, 100, 10, 1 and, 0.1 spore equivalent concentrations.

Bd TaqMan Assay

The TaqMan assay is a probe-based qPCR method that uses a sequence-specific, fluorescence-labelled probe added to the Bd primer mix. The assay designed by Boyle et al. (2004) was optimised for the Bioline SensiFast Probe Lo-Rox qPCR kit (Total Lab Systems, catalogue no. BIO-84020). The optimised reagent master mix was composed of 5 μL 2 \times Bioline SensiFAST Probe Lo-Rox reagent, 0.5 μL 20 \times TaqMan® Gene Expression Assay (Applied Biosystems, catalogue no. 4332079), and 4 μL DNA template (1:2 diluted with TE buffer, pH 8) or DNA standard. The probe/primer mix was composed of 900 nM of the ITS1-3 and 5.8 Chytr primers and 250 nM Chytr MGB2 (probe). The TaqMan assay was run using the

following thermal conditions: polymerase activation at 95°C for 3 m followed by 50 cycles of denaturation at 95°C for 5 s, annealing at 59°C for 10 s and extension at 72°C for 30 s. All assays were performed in triplicate.

Randomly selected DNA samples that yielded positive results were verified using 2% agarose gel electrophoresis. Amplicons from Bd-positive DNA samples were purified using MEGAquick-spin plus Total Fragment DNA Purification kit (iNtRON, catalogue no. IN17290), following manufacturer's instructions. Purified amplicons were sent to the Genetic Analysis Services, Department of Anatomy, University of Otago for sequence confirmation by capillary sequencing. Sequences were analysed using Geneious software with the basic local alignment search tool (BLAST). Sequences were aligned and a phylogenetic tree was constructed using the neighbour-joining method in Mega X (version 10.1.8, Kumar et al. 2018).

The Bd status of each frog was based on the result of the TaqMan assay. The amplification of two or three (all) replicates was classified as a Bd-positive frog. If only one of the three replicates yield an amplification, the TaqMan assay was repeated in triplicate. If the result was the same, one out of three replicates showed amplification, it was considered Bd-weakly-positive. When none of the three replicates were positive, the frog was classified as Bd-negative. The number of Bd DNA copies per swab was calculated by multiplying the qPCR-generated DNA copy count with the DNA template dilution factor and volume of DNA template in the master mix then multiplied by the concentration of DNA (Maier & Peterson 2016).

Statistical Analysis

Data analysis was carried out in R Statistical Software (version 4.1.1, R Core Team 2021). To determine whether the number of Bd DNA copies differed between frog species and location in the wild and captive populations, a Kruskal-Wallis test (a non-parametric test) was used. For this analysis, the number of Bd DNA copies per swab was the response variable, and species (*L. archeyi*, *L. hamiltoni*, or *L. hochstetteri*) and location (wild populations: Whareorino Forest, Pukeokahu, Maud Island; captive populations: Auckland Zoo, Zealandia, and University of Otago) were the predictor variables.

For body condition, a linear model was used to test for a relationship between Bd presence and sampling location (predictor variable) and scaled mass index (response variable), separately for each species where Bd was detected. A chi-square test was used to compare the prevalence of Bd between sampling locations for *L. archeyi* in Whareorino Forest and Pukeokahu (wild populations) and *L. hamiltoni* in University of Otago and Zealandia (captive populations). *Leiopelma hochstetteri* was not included in the analysis since it was sampled only in one location.

To understand whether the likelihood of a frog being Bd-positive differed between the sexes, the number of infected and uninfected frogs were compared between males and females using a chi-square test. Only *L. hamiltoni* from the captive population was included in this analysis as they were the only ones of known sex and detected to be Bd-positive. Prevalence of Bd in relation to sex in captive *L. hochstetteri* and *L. archeyi* were not measured as no individual was detected with Bd.

A Kruskal-Wallis test was used to identify if the number of Bd DNA copies in *L. archeyi* in Whareorino differed between years (2014–2020). In addition, a chi-square test was used to compare the prevalence of Bd in *L. archeyi* in Whareorino between 2014–2020.

Results

Prevalence of Bd on native *Leiopelma* frog species in select New Zealand frog locations

A total of 333 skin swabs from 324 frog individuals were screened for the presence of Bd (see Appendix S2). Twenty-two of the 324 frogs swabbed were Bd-positive (6.8%) and a further two frogs were classified as weakly-positive (*L. hamiltoni* sampled from Maud Island; Fig. 1). A complete list of all the frogs swabbed and their corresponding Bd status is presented in Appendix S3. Bd was detected on *L. archeyi* in Whareorino Forest and *L. hamiltoni* at the University of Otago. The prevalence of Bd in *L. archeyi* (wild) and *L. hamiltoni* (captive) was not significantly different between locations (*L. archeyi*: $\chi^2 = 0.67$ df = 1, $P = 0.41$; *L. hamiltoni*: $\chi^2 = 2.15$, df = 1, $P = 0.14$). All the sampled *L. hochstetteri* tested Bd-negative. The Bd DNA copies per swab were not significantly different across frog species (Kruskal-Wallis $\chi^2_1 = 0.06$, $P = 0.80$) or locations (Kruskal-Wallis $\chi^2_2 = 1.76$, $P = 0.42$) in the wild populations. In Whareorino Forest, one *L. archeyi* skin swab contained 458 519 Bd DNA copies (Appendix S4). For the captive populations, the Bd DNA copies per swab were also not significantly different across frog species (Kruskal-Wallis $\chi^2_2 = 2.16$, $P = 0.34$) or locations (Kruskal-Wallis $\chi^2_2 = 4.56$, $P = 0.10$). Frogs sampled in this study did not exhibit any clinical signs of chytridiomycosis.

Bd-positive qPCR products from *L. archeyi* and *L. hamiltoni* produced bands of approximately 146 bp which

is the expected band size for Bd DNA (Appendix S4). The same band size was also observed on positive standards at Bd 10 spore μL^{-1} and Bd gBlock 1000 DNA copies μL^{-1} which was consistent with amplification observed using the TaqMan assay was due to the presence of Bd DNA from the skin swabs. Sequence analysis using a neighbor-joining tree showed the phylogenetic relationship of the selected Bd-positive samples from *L. archeyi* and *L. hamiltoni* both aligned with the standard used (gBlock) clustering with Bd JEL 197 and the local Bd strain RTP6 (Fig. 2).

Patterns of Bd prevalence with sex and body condition

In captive *L. hamiltoni*, only male frogs were detected with Bd ($n = 3$; Bd-negative $n = 6$; $\chi^2 = 5.37$, df = 1, $P = 0.021$) (Fig. 3) but further study is necessary to confirm this pattern more widely.

The presence of Bd has no significant influence on the body condition of *L. hamiltoni* or *L. archeyi* (Fig. 4). Bd status has no significant effects on the scaled mass index of *L. archeyi* in Whareorino Forest ($F_{1,199} = 1.24$, $P = 0.267$), and *L. hamiltoni* in the University of Otago ($F_{1,21} = 0.01$, $P = 0.914$) or on Maud Island ($F_{1,16} = 4.13$, $P = 0.059$).

Temporal trends in the Bd prevalence in the Whareorino Population

The overall prevalence of Bd in *L. archeyi* in Whareorino Forest was 9.2% (19/206) (Appendix S6). The number of Bd DNA copies per swab (including negative swabs) was not

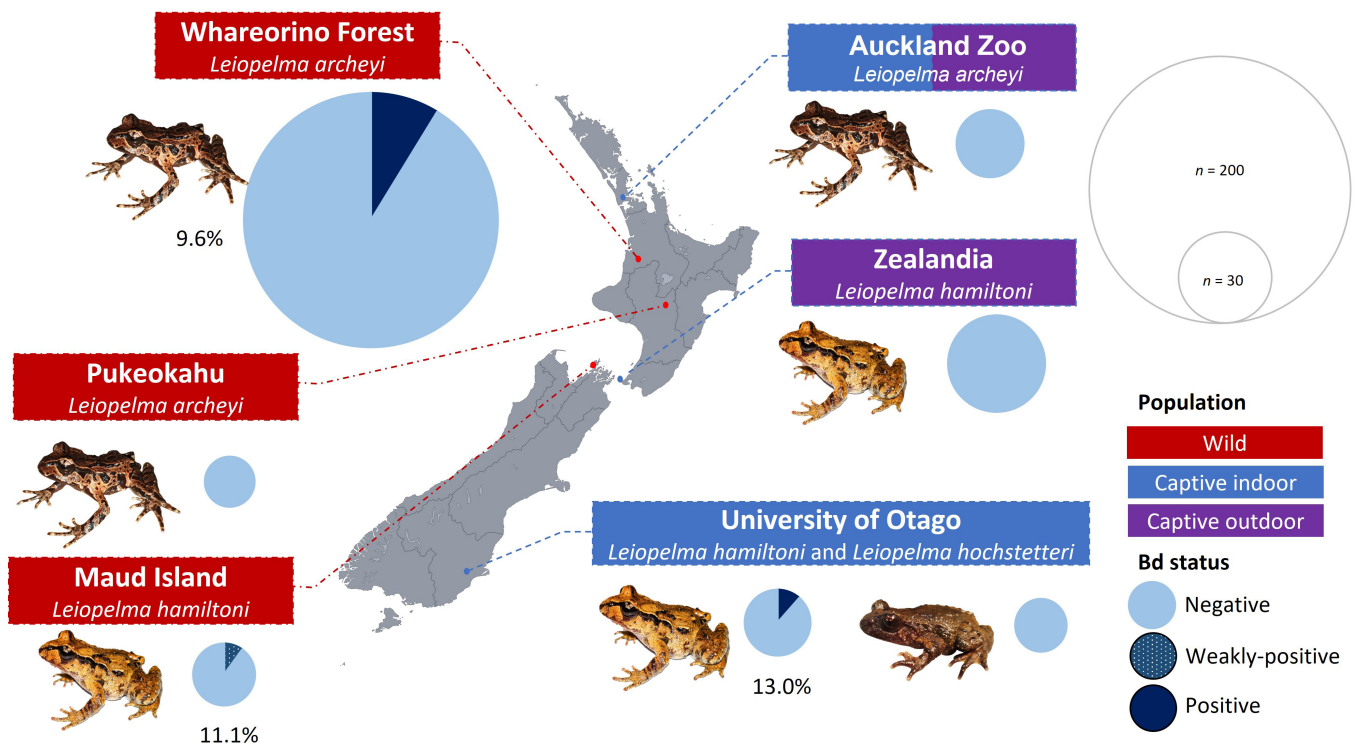


Figure 1. Sampling locations and *Batrachochytrium dendrobatidis* (Bd) prevalence of native frog species collected in this study. The three species of native frogs that were captured and swabbed for Bd screening were *L. archeyi*, *L. hamiltoni*, and *L. hochstetteri*. The locations with red labels represent the wild populations, blue labelled locations represent the captive indoor populations and purple for captive outdoor populations. The prevalence of Bd presence is represented by pie charts where orange represented the percentage of Bd-negative, yellow for Bd-positive, and green for Bd-weakly-positive frogs. The outlined circles in the top right corner refer to the sample sizes based on circle size.

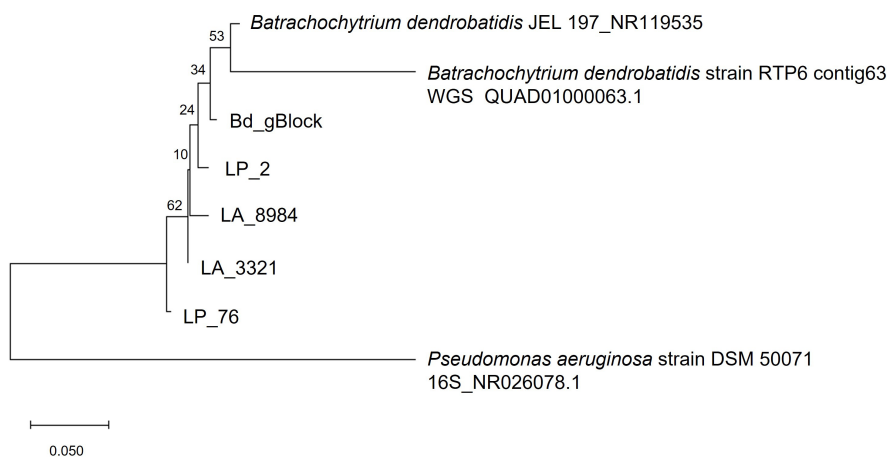


Figure 2. Phylogenetic tree of selected amplicon sequences from *Batrachochytrium dendrobatidis* (Bd) positive samples and the Bd gBlock. Bd-positive samples: *Leiopelma archeyi* (LA_3321, LA_8984), *L. hamiltoni* (LP2) from Maud Island and (LP_76) from University of Otago, and standard (Bd gBlock) clustering with Bd JEL 197 and Bd strain RTP6 while *Pseudomonas aeruginosa* was used as an outgroup.

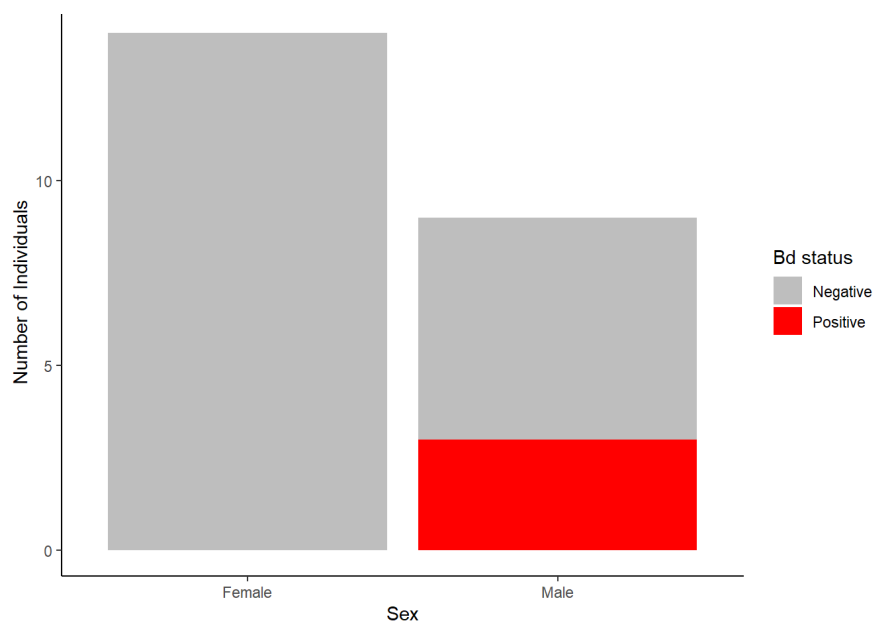


Figure 3. Prevalence of *Batrachochytrium dendrobatidis* (Bd) positive *Leiopelma hamiltoni* for sex-identified individuals from the captive population at the University of Otago.

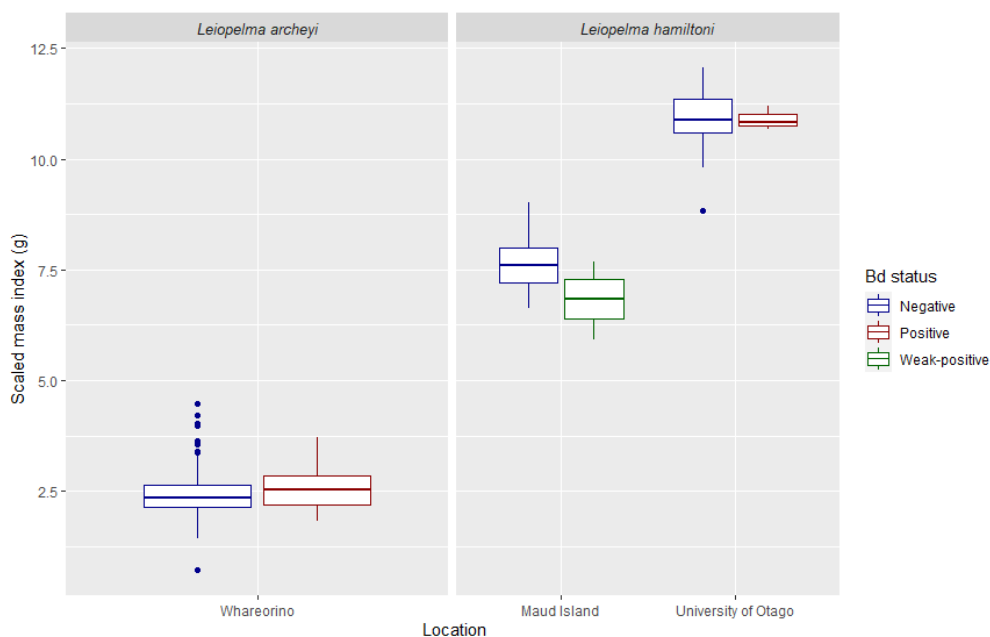


Figure 4. Body condition of the *Leiopelma archeyi* and *L. hamiltoni* in relation to *Batrachochytrium dendrobatidis* (Bd) status and sampling location.

significantly different between years (Kruskal-Wallis $\chi^2_6 = 11.89, P = 0.06$). The number of Bd-positive *L. archeyi* did not differ significantly between years ($\chi^2 = 9.68, df = 6, P = 0.14$).

Discussion

Our results showed that Bd still persists in New Zealand's native frogs, in spite of their demonstrated ability to clear Bd infection following experimental laboratory exposure (Shaw et al. 2010; Ohmer et al. 2013). This study provides important insights into the current status of Bd among native leiopelmatid frogs across New Zealand.

Prevalence of Bd in native frogs

Twenty years since its discovery Bd was still present in *L. archeyi* in Whareorino forest, in *L. hamiltoni* at the University of Otago, and on Maud Island at a generally low prevalence (6.8%, 22/324). The prevalence of Bd did not significantly differ across the six sampled locations (Fig. 1). The low mortality rate of native frogs in relation to Bd suggests that Bd is endemic to New Zealand (Shaw et al. 2013). Kruger (2020) highlighted that detection of Bd DNA from a frog sample using qPCR does not necessarily equate to infection intensity or viability of Bd to cause infection. No clinical symptoms were found among the Bd-positive individuals tested in this project, but this does not rule out chytridiomycosis. Long-term observational monitoring would be required, including collection of frog skin swabs to observe potential changes in the prevalence of disease.

Leiopelma archeyi was sampled from three sites including Auckland Zoo, Whareorino Forest, and Pukeokahu Forest. Auckland Zoo is a breeding facility for *L. archeyi*, and all frogs from Auckland Zoo have consistently tested negative for Bd. These frogs were originally translocated in 2016 from Coromandel and Whareorino populations which are both Bd-infected sites (Bell et al. 2004; Bishop et al. 2009; Shaw et al. 2013). The Bd prevalence of *L. archeyi* in Whareorino forest was 9.2% (19/206) between 2014–2020, slightly higher than the 6.0% Bd prevalence measured between 2005–2010 (Shaw et al. 2013).

The highest number of Bd DNA copies among Bd-positive individuals was a *L. archeyi* (FrogID A232) from Whareorino forest collected in 2018 with 458 519 Bd DNA copies per swab. The strain of Bd found in the Bd-positive leiopelmatid frogs clustered with Bd strain JEL197 (Fig. 3). JEL197 is part of the virulent Global Pandemic Lineage (GPL) that was isolated from a blue poison dart frog that died because of chytridiomycosis in National Zoological Park, Washington, USA (Longcore et al. 1999). The equivalent copy number per zoospore for Bd JEL197 is 169 copies per zoospore (Kirshtein et al. 2007). Following this conversion, 458 519 Bd DNA copies is equivalent to 2713 zoospores per swab. This count is below the infection intensity threshold suggested by Vredenburg et al. (2010) called Vredenburg's "10 000 Zoospore Rule" suggesting infection titre is not sufficient to cause disease. Vredenburg et al. (2010) suggested that chytridiomycosis could result in amphibian decline if the population has a mean infection intensity of 10 000 zoospore equivalent per swab (Vredenburg et al. 2010; Kinney et al. 2011).

Currently, chytridiomycosis is not considered a major threat to *L. archeyi* because their ability to clear the infection has been demonstrated following experimental exposure

in the laboratory (Bishop et al. 2009; Shaw et al. 2010). *Leiopelma archeyi* in Pukeokahu that were swabbed were Bd-negative. Pukeokahu is a translocation site for 70 *L. archeyi* originally from Whareorino Forest. Frogs were translocated to Pukeokahu in 2006 to reduce the risk of chytridiomycosis in the *L. archeyi* population. An additional 60 individuals were translocated in 2016 to increase genetic diversity of the original translocated frogs (Sherley et al. 2010; Cisternas 2019). All of these translocated frogs were Bd-negative at the time of release (Cisternas 2019) and appear to have retained their negative status to date.

Batrachochytrium dendrobatidis was detected in the captive populations at the University of Otago but not in Zealandia Ecosanctuary. All *L. hamiltoni* housed at the University of Otago and Zealandia were translocated from predator-free Maud Island (Bishop et al. 2013). Captive frogs at the University of Otago were previously experimentally infected with a New Zealand Bd strain (RTP4) in 2009 to explore the virulence of this strain to *L. hamiltoni*, *L. hochstetteri* and *Li. ewingii* (Ohmer et al. 2013). In New Zealand, Bd was successfully isolated from an infected *Li. ewingii* in Dunedin (Shaw et al. 2014; Sumpter et al. 2018). In one study the strain was named RTP6 and a whole genome analysis confirmed that it belongs to the highly virulent BdGPL (Global Pandemic Lineage) strain (Sumpter et al. 2018). Using BLAST and the National Center for Biotechnology Information (NCBI) databases, the strain of Bd that we found in the current study also aligned to the local strain RTP6 and JEL197. JEL197 is a BdGPL strain that is highly deadly to most species of frogs worldwide (Antwis et al. 2015).

Leiopelma hochstetteri is the most widely distributed native frog species and is the only species that has never been found to be Bd-positive (Thurley & Haigh 2008; Moreno et al. 2011; Bishop et al. 2013). As for previous studies, all *L. hochstetteri* kept at the University of Otago tested negative for Bd despite being the only semi-aquatic species of the three native frogs. Association with aquatic environments for breeding may pose more threat of chytridiomycosis since Bd releases its zoospores in the water to search for and infect a new host (Kriger & Hero 2007). One possible explanation for this immunity is the presence of antimicrobial peptides and skin bacteria that may play a role in protecting these frogs against chytridiomycosis (Melzer & Bishop 2010). The temperature at the time of sampling may have influenced the detection of Bd in the native frogs. For the captive populations, frogs were swabbed between December 2019 and February 2021 when the temperature was between 16.0–18.0 °C. Frogs from the wild populations were collected during the spring of 2014 and 2020 where the temperature ranged between 6.2–16.0 °C during time periods when frogs were sampled. The average temperature during the sampling periods was 10.3 °C, favourable for Bd growth. Higher Bd prevalence has been observed in colder seasons (Berger et al. 2004). Bd can grow between 4 and 25 °C with an optimum temperature between 17 and 25 °C (Fisher et al. 2009) and the virulence typically is at its highest between 12 and 23 °C (Berger et al. 2004; Shaw et al. 2013). Bd is sensitive to high temperature (37 °C; Johnson et al. 2003). Season and temperature may influence the presence of Bd in frogs and these factors should be considered when detecting Bd in wild populations. Sampling in months when the temperature is ideal for Bd growth would maximise detection in a population with very low prevalence.

Relationship between body condition and Bd prevalence

The difference in body condition between wild and captive *L. hamiltoni* and *L. archeyi* may be associated more with feeding behaviour in their respective habitats and not Bd status. Bd-positive frogs in this study seemed to have heavier mass than Bd-negative frogs but these frogs were also found in captivity. Bd-infected frogs sometimes manifest symptoms such as lethargy, loss of appetite, and loss of righting reflex (Kolby & Daszak 2016). Loss of weight in relation to inappetence is one side effect of chytridiomycosis. In this study, Bd-positive *L. hamiltoni* had a higher scaled mass index than Bd-negative *L. hamiltoni*. Bd-positive *L. hamiltoni* were sampled from a captive population at the University of Otago. A similar pattern was observed in *L. archeyi* from Auckland Zoo where they have significantly higher scaled mass index when compared to the wild populations: *L. archeyi* in Whareorino Forest and Pukeokahu, and *L. hamiltoni* on Maud Island (Appendix S5). This result suggests captivity is the main factor that influences the difference in body condition in *L. hamiltoni* and *L. archeyi*. Frogs in the wild need to hunt for their prey and would eat any live insects or animals that they may encounter whereas frogs in captivity are fed reliably with live insects on a weekly basis. The environmental condition for captive frogs is set to their optimal growth requirements, is stress-free with no predators and provides regular assessment of their health and condition to guarantee survival of captive frogs (Davis & Maerz 2011). The feeding pattern and favourable environment for the captive frogs most likely contributed to the difference of scaled mass index of leiopelmatid frogs rather than Bd presence.

Temporal trends in Bd prevalence in the Whareorino population

The reasons for the temporal changes in Bd prevalence and infection load in Whareorino are unclear. Average air temperatures remained similar between years, so it seems unlikely that changes in temperature are driving these patterns. The higher Bd prevalence in 2017 and 2018 may be associated with rainfall during the sampling periods. Increased precipitation has been linked to higher Bd prevalence as observed in amphibians in Oklahoma (Watters et al. 2019) and in anurans in the Brazilian Atlantic Forest (Ruggeri et al. 2018) where frog populations have been declining due to Bd. For terrestrial frog species like *L. archeyi*, water accumulation during rainy seasons may promote dispersal of zoospores that potentially infect more individuals and deposit greater concentrations of zoospores on leaves, rocks, and other surfaces (Berger et al. 2005; Ruggeri et al. 2018). The reasons behind these temporal changes in infection need further investigation. Sampling and swabbing of frogs across different seasons would provide a better understanding of the influence of temperature, rainfall, and other abiotic factors on the prevalence of Bd across the country. Sampling in months when the temperature is ideal for Bd growth would maximise the sampling efforts in a population with very low prevalence. This would also provide knowledge to improve the conservation actions and monitoring of Bd on native frogs in the wild.

Conservation concerns

A concern raised by this project is the presence of Bd weakly-positive results from Maud Island. Based on the survey conducted by Bell (2010), 60 individuals of *L. hamiltoni*—referred to as *L. pakeka* in Bell 2010—swabbed in 2006 and 2008 were all Bd-negative. The Bd-weakly-positive may be

associated with the specificity of the TaqMan assay. Skerratt et al. (2011) suggested that an amplification of one of three replicates to be indeterminate but considering the sensitivity of the test, it can be declared as either positive or negative. Kruger (2020) stated that amplification of one well can be considered as positive when the same result (one out of three amplification) was obtained after a second run. The Bd weakly-positive detected in swabs from Maud Island was confirmed when the assay was re-run. Strict procedures have been implemented by the Department of Conservation in areas where frogs are found to prevent the possible spread of chytridiomycosis, including sanitation and quarantine procedures required before any study or survey can be conducted in New Zealand's frog protected areas. Despite this, Bd was still detected in the isolated population of *L. hamiltoni* on Maud Island. It is now important to re-assess and swab the frogs on Maud Island to confirm the presence and to evaluate the potential threat of Bd in the *L. hamiltoni* population.

The first case of chytridiomycosis in native frogs (*L. archeyi*) in New Zealand was found in Coromandel in 2001 (Bell et al. 2004). *Leiopelma hochstetteri* also live in this region but were never found to be Bd-positive. Unfortunately, *L. hochstetteri* and *L. archeyi* were unable to be sampled in the Coromandel Peninsula in this study. Consequently, the current Bd prevalence of the wild population of *L. hochstetteri* remains unknown, and the current prevalence of Bd in *L. archeyi* on the Coromandel Peninsula could not be confirmed.

As a conclusion, chytridiomycosis may not pose an immediate threat to native leiopelmatid frog species, since all the *Leiopelma* species appear to have low susceptibility to this disease. Bd prevalence on wild and captive animals was not significantly different and prevalence of Bd did not differ between sampling locations, frog species, or population. Captive *L. archeyi* and *L. hamiltoni* tended to have significantly higher scaled mass index than their wild counterparts suggesting that the difference in their body condition was associated to the feeding pattern of frogs in captivity. In addition to a continual supply of reliable food, captive frogs have a very small foraging space and are likely less active.

An in-depth analysis (e.g. whole genome sequencing) of the Bd strain infecting native frog species to assess its virulence and potential threat to non-endemic frogs could prove enlightening for providing information on the origin and mode of transmission of Bd and disease mechanisms in general. We also recommend continual surveillance of Bd prevalence in the Whareorino population to address conservation efforts necessary to safeguard the *L. archeyi* population.

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Conflicts of interest: The authors declare no conflicts of interest.

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Supplementary material

Additional supporting information may be found in the supplementary material file for this article:

Appendix S1. Complete sequence of *Batrachochytrium dendrobatidis* gBlock gene fragment prepared by IDT Asia in Singapore.

Appendix S2. Prevalence of *Batrachochytrium dendrobatidis* in three native frog species *Leiopelma hamiltoni*, *L. archeyi*, and *L. hochstetteri* in captive and wild populations across six sites in New Zealand.

Appendix S3. Frog individuals collected in different sampling locations that were declared with Bd-positive, Bd-negative and Bd-weakly-positive status.

Appendix S4. Agarose gel electrophoresis of selected Bd-positive samples was performed to confirm the amplicon observed was the expected size for Bd DNA.

Appendix S5. The scaled mass index (g) of the three endemic frog species (*Leiopelma archeyi*, *L. hamiltoni* and *L. hochstetteri*) in captive and wild populations in relation to their Bd status and location.

Appendix S6. Temporal trends in the *Batrachochytrium dendrobatidis* (Bd) prevalence and abundance in *L. archeyi* in Whareorino Forest sampled between 2014–2020.

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