1 Title: Decline and re-expansion of an amphibian with high prevalence of chytrid fungus

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Abstract

The disease chytridiomycosis, caused by the fungal pathogen *Batrachochytrium dendrobatidis* (Bd), is a key driver of global amphibian declines. While chytridiomycosis can cause extinction, many susceptible species persist after an initial period of decline, albeit with reduced abundance and distribution. Emerging evidence indicates that amphibian abundance can recover within remnant populations, but to date, the capacity of amphibian populations to re-expand into historically occupied habitat has received limited research attention. We surveyed 145 sites in 2011 and 2012 to determine if populations of the whistling tree frog (*Litoria verreauxii verreauxii)* have re-expanded compared with historical data from 1975-6, 1990 and 1996. *L. v. verreauxii* underwent a major range contraction likely caused by chytridiomycosis between the first two time periods. Populations have recently re-expanded, with 39 new sites colonised despite high prevalence of Bd. We suspect that changes in disease dynamics have resulted in the increased coexistence of *L. v. verreauxii* and Bd. Habitat attributes at sites that retained frogs for the duration of the study indicate that high quality habitat may contribute to buffering against population level effects of Bd. Colonised sites had more coarse woody debris, suggesting a possible habitat management strategy to encourage range expansion for this species. Given sufficient time and adequate source populations in high quality habitat, it is possible that other amphibian species may re-expand from chytridiomycosis-induced declines. This provides an impetus for the protection of historical, but currently unoccupied amphibian habitats and highlights the importance of maintaining high quality habitat to help species survive novel shocks such as pandemic diseases.

1. Introduction

In an era of rapid biodiversity loss, amphibians are especially vulnerable (Stuart et al., 2004) due to multiple threats, including habitat destruction and emerging infectious disease (Wake and Vredenburg, 2008; Hof et al., 2011). One of the most pressing threats is the pandemic chytridiomycosis (Wake, 2012), an infectious skin disease caused by the fungal pathogen *Batrachochytrium dendrobatidis* (hereafter Bd) (Berger et al., 1998) that has caused the decline or extinction of at least 200 species (Skerratt et al., 2007). Chytridiomycosis has resulted in "the most spectacular loss of vertebrate biodiversity due to disease in recorded history" (Skerratt et al., 2007) and provides a devastating example of the threat posed by emerging infectious diseases to biodiversity (Fisher et al., 2012).

Although chytridiomycosis has caused the extinction of many species, the majority of susceptible species experience range contractions but subsequently persist with enzootic Bd infection, albeit with greatly reduced distribution and abundance (Walker et al., 2010; Puschendorf et al., 2011). Disease dynamic models suggest that population recovery is possible in populations with enzootic Bd (Briggs et al., 2010). Recently, Newell et al. (2013) demonstrated that two populations of the endangered *Mixophyes fleayi* have experienced sustained population growth and this combined with observations of increased abundance in several *Litoria serrata* (Syn. *L. genimaculata*) populations (McDonald et al., 2005; Richards and Alford, 2005), indicates that the recovery of at least some remnant populations of these species is occurring. However, despite the extensive Bd literature (Muths et al., 2011), evidence documenting range re-expansion is limited to brief observations from northern Australia described by McDonald (2002) and McDonald et al. (2005). Documenting evidence of range re-expansion would greatly aid our understanding of the long-term response of wildlife to novel diseases, and provide an impetus for the protection of historical, but currently unoccupied habitat.

Here, we examine the long-term response of an amphibian species approximately three decades after the emergence of Bd in our study region. *Litoria verreauxii verreauxii* (whistling tree frog) experienced severe declines in upland areas of the Southern Highlands of eastern Australia in the 1980s (Osborne, 1989, 1990, 1992; Osborne and Hunter, 1998). Prior to its decline, *L. v. verreauxii* was considered ubiquitous across the region (Osborne, 1989, 1990, 1992), as demonstrated by non-targeted historical surveys in 1975-1976 that detected the species at 73 of 79 sites (M.J. Littlejohn, University of Melbourne, unpublished results).

Although a direct link between the decline of *L. v. verreauxii* and Bd in the Southern Highlands has not been demonstrated, several lines of evidence indicate that the emergence of Bd provides the most plausible explanation for the decline. First, the decline of *L. v. verreauxii* occurred in the early 1980s and coincided with the local extinction of four co-occurring species (*Litoria aurea, Litoria castanea, Litoria raniformis* and *Pseudophryne bibronii*) (Osborne, 1990, 1992; Osborne and Hunter, 1998; Hamer et al., 2010). The rapid nature of these declines is consistent with the decline of other species for which chytridiomycosis has been implicated (Berger et al., 1998; Lips et al., 2006; Vredenburg et al., 2010). Disease is a likely cause of mass mortality of adults and the spatiotemporal nature of the decline is consistent with a spreading infectious disease (Laurance et al., 1996; Skerratt et al., 2007). Second, retrospective screening of museum *P. corroboree* and *P. pengilleyi* specimens collected from the Southern Highlands failed to detect Bd prior to 1980, but found Bd was common in specimens collected after populations began to decline (Hunter et al., 2010). Third, experimental work on the closely related subspecies *L. verreauxii alpina* (recent genetic analysis does not support distinguishing this sub-species; L. Price 2012, University of Newcastle, personal communication) has demonstrated very high susceptibility to Bd under laboratory conditions (S. Cashins 2013, James Cook University, personal communication), indicating that *L. v. verreauxii* is susceptible to Bd. Lastly, the species has been killed by chytridiomycosis in the wild (Berger et al., 2004).

To study long-term changes in *L. v. verreauxii* occupancy, we used data collected from three time periods; 1975-6 before Bd, 1990 and 1996 shortly after Bd arrival, and 2011 and 2012. We were interested in 1) confirming the decline of *L. v. verreauxii* between the first two survey periods and 2) whether *L. v. verreauxii* populations have expanded between surveys from the 1990s compared to surveys from 2011 and 2012, and if so, to what extent. We also investigated whether recent changes in occupancy are affected by breeding habitat variables and quantified the current prevalence of Bd in persistent and recently colonised populations.

2. Methods

2.1. Study area

We conducted our study in the Southern Highlands region of south-eastern Australia (Fig. 1). The region has a temperate climate with an average winter minimum of 0.6°C and a maximum of 12.2°C and corresponding summer averages of 12.5°C and 27°C (BOM, 2012). Rainfall is consistent throughout the year with an annual average of 616 mm, however, rainfall can be greatly reduced during infrequent El Nino droughts (BOM, 2012). We surveyed 145 sites located in grazing, suburban and protected landscapes. A range of wetland habitat types were surveyed including ponds (n = 94), lake shores (n = 3) and small streams (n = 48). All sites superficially resembled suitable breeding habitat (Anstis, 1976).

2.2. Study species

From late winter through spring, breeding aggregations of *L. v. verreauxii* use a range of habitats including ponds, creeks and swamps (Anstis, 1976). Males call from aquatic vegetation or on adjacent banks and eggs are deposited below the water surface attached to aquatic vegetation (Anstis, 1976).

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121 *2.3. Frog surveys*

122 This study is based on frog surveys carried out in 1975-76 ("historical surveys") by M. J. Littlejohn
123 (University of Melbourne), 1990 and 1996 ("baseline surveys") by F.G. and W.O. and 2011 and 2012
124 ("current surveys") by B.S. The location of historical surveys was determined by reviewing the field
125 notes of M.J. Littlejohn who undertook extensive surveys throughout the study region in 1975-1976.
126 Using a topographical map, we were able to identify the specific locations of 23 of the sites surveyed
127 by Littlejohn (Fig. 1). To increase the number of sites sampled and provide a robust baseline we
128 selected an additional 122 sites in the immediate vicinity of the historical surveys that form the
129 baseline surveys. These sites were surveyed in 1990 (n = 20) and 1996 (n = 125). In 1990, surveys
130 were conducted on overcast nights or following rain and in 1996, a reference site was used to check
131 male calling activity prior to survey.

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133 In 2011, all sites were surveyed three times during August and September. The duration of each
134 survey was five minutes. Air temperature, relative humidity, wind speed, time to *L. v. verreauxii*
135 detection and the presence of other amphibian species were recorded. Cognizant of concerns
136 associated with quantifying changes in amphibian site occupancy (see Pechmann et al., 1991; Alford
137 and Richards, 1999), we repeatedly surveyed sites in 2011 to calculate detectability and resurveyed
138 sites once in 2012 to quantify inter-annual variation in site occupancy. Furthermore, to avoid
139 concerns associated with inferring change from presence only records (Skelly et al., 2003) we ensured
140 that our baseline surveys included a large number of presence and absence sites. All surveys were
141 auditory (*L. v. verreauxii* has a clear, loud, easily distinguishable call) and were conducted during the
142 breeding season to maximise likelihood of detection and surveys were not conducted during high
143 winds or heavy rain.

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146 *2.4.* Batrachochytrium dendrobatidis *sampling*

147 Sampling was conducted to investigate if re-expansion had occurred in the ongoing presence of Bd
148 and if Bd was present, to quantify prevalence and intensity of infection. During the 2011 breeding
149 season we used sterile swabs (Medical Wire & Equipment Co. MW 100–100) to sample 65 adult *L. v.*
150 *verreauxii* at four sites (see Fig. 1). Each sample was collected in a standardised way with three
151 strokes on each side of the abdominal midline, the inner thighs, hands and feet. Samples were
152 analysed by a commercial lab (cesar, Melbourne, Australia) using real-time quantitative PCR
153 following the methodology of Boyle et al. (2004) and Hyatt et al. (2007) with the exception that
154 samples that initially returned equivocal results were re-analysed using a Qiagen master mix instead
155 of the Taqman master mix. We considered a sample positive if all three wells returned a positive
156 reaction. After re-analysis, one sample returned one out of three wells positive and was classified as
157 equivocal.

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159 *2.5. Habitat measurements*

160 In October 2011 the following information was collected at all sites: percentage cover of emergent
161 vegetation, percentage of the riparian zone (0-2 m from the water's edge) with no ground cover (bare
162 bank), percentage with tussock cover over 50 cm and the number of pieces of coarse woody debris (>
163 1 m x 10 cm diameter). Emergent vegetation, ground and tussock cover were measured because they
164 are significant predictors of amphibian presence within the study region (Hazell et al., 2001). All
165 percentage values were visual estimates, which have been used effectively within the study region
166 (Hazell et al., 2001).

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169 *2.6. Statistical analysis*

170 To investigate whether changes in site occupancy were related to habitat, we classified sites based on
171 frog presence/absence during baseline and 2011 surveys. *Present* sites supported frogs in both
172 periods (n = 22), *colonised* sites did not have frogs during baseline surveys but frogs were present in
173 2011 (n = 34) and *absent* sites did not support frogs in either period (n = 86). Although *L. v.*
174 *verreauxii* is highly detectable in the study region (single visit probability of detection 0.92,
175 increasing to 0.99 after three surveys), it is possible that individuals were present, but not detected at a
176 small number of absent sites, potentially resulting in the misclassification of some absent sites. Three
177 sites that supported frogs during the baseline surveys but did not support frogs in 2011 were excluded
178 from the analysis because of low sample size (n = 3). These sites were spatially clustered (Fig. 1) and
179 because habitat appeared suitable, we cannot rule out the role of disease in driving these extinctions.
180 Conservatively, we use the term colonised to describe sites that went from absent to present, however,
181 given the historical distribution of *L. v. verreauxii* in our study region, colonised sites likely represent
182 *re*colonised sites.

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184 We used multinomial generalised linear modelling to investigate differences in habitat characteristics
185 among site types. Prior to analysis we ensured that habitat variables were not correlated. The number
186 of pieces of coarse woody debris was natural log +1 transformed to ensure that the variance of the
187 residuals was constant across the range of fitted values (Quinn and Keough, 2002). Percentage of the
188 riparian zone with no ground cover and percentage of emergent vegetation exhibited bimodal
189 distributions and were converted to binary data. Percentage of tussock cover remained highly skewed
190 after transformation and was also converted to a binary data. The three variables converted to binary
191 data were split at ≥ 50% cover, representing high and low cover classes. Fifteen candidate models
192 arising from all combinations of the four explanatory variables were constructed. We used an
193 information-theoretic model selection process to rank models based on their Akaike's Information

Criterion value with a correction for small sample size using the package 'AICmodavg' (Mazerolle, 2013) . All analyses were completed in R 2.10.0 (R Development Core Team, 2009).

Using repeat survey data from 2011 we investigated the adequacy of the survey effort to reliably detect frog presence/absence. First, we examined the effects of temperature, humidity and wind on probability of detection. All weather variables were shown to be non-significant ($P > 0.05$) and were not considered further. Second, we calculated the probability of detecting frog presence after a single visit (MacKenzie et al., 2002). We then used formulae from Wintle et al. (2005) to calculate the cumulative probability of detecting the species following three visits assuming that the species was present. We used the 'unmarked' package (Fiske and Chandler, 2011) in R 2.10.0 (R Development Core Team, 2009) for all detectability analyses.

3. Results

3.1. Decline and expansion

All 23 sites surveyed in 1975-76 were occupied by *L. v. verreauxii*. During the baseline surveys seven of 23 historical sites were occupied, increasing to 11 of 23 during current surveys. Between the baseline surveys and 2011 *L. v. verreauxii* site occupancy increased from 25 of 145 to 56 of 145 (Fig. 1). In 2012, five additional sites were colonised (Fig. 1). These sites were located close to sites where frogs were detected in 2011 (Fig. 1). Three sites that did not support frogs in the baseline surveys, but supported frogs in 2011 did not support frogs in 2012 (Fig. 1). These sites only supported very small numbers of frogs in 2011. In 2011 and 2012 frogs were present at all but three sites where they were detected in baseline surveys. In total, 39 sites were colonised between the baseline surveys and 2011-12, while frogs were extirpated at only three sites.

3.2. Detectability

Over the four surveys in 2011 and 2012 *L. v. verreauxii* was recorded 215 times. At occupied sites, *L. v. verreauxii* was generally detected in the first minute of survey, with time to detection less than one minute on 91.6% of occasions (<2min. 96.2%, 3<min. 98.6%, <4min. 100%). At sites surveyed three times in 2011, the probability of detecting frogs on any single visit was 0.92 (*S.E.* = 0.02) and after three visits the probability of detecting frogs given presence was 0.99 (*S.E.* = 0.02).

3.3. L. v. verreauxii *habitat analysis*

The best supported model contained three explanatory variables; percentage of the riparian zone with no ground cover, percentage with tussock cover and the number of pieces of coarse woody debris. The absence of frogs during both baseline and current surveys was positively associated with the amount of bare bank at a site (Table 1). The presence of frogs during baseline and current surveys was positively associated with tussock cover. Site colonisation was positively associated with coarse woody debris (Table 1).

3.4. Bd in L. v. verreauxii *populations*

In 2011, infection prevalence in sampled *L. v. verreauxii* adults was 80% (Table 2). For infected frogs, the mean infection intensity was 9267 (*S.E.* = 4635) zoospore equivalents and the median was 704 zoospore equivalents (Table 2). While sampling, four frogs exhibited signs of severe chytridiomycosis (most noticeably, loss of righting reflex, Voyles et al., 2009) and two recently dead frogs were found in calling positions.

4. Discussion

The capacity of amphibian populations to recover from chytridiomycosis-driven declines is poorly understood. To our knowledge, we provide the first systematic documentation of the decline and

subsequent large-scale re-expansion of a susceptible species following widespread extirpation most likely caused by chytridiomycosis. It is possible that there is publishing bias towards documenting ongoing population impacts (e.g. Murray et al., 2009; Phillott et al., 2013) compared with partial recovery of populations from chytridiomycosis (e.g. McDonald, 2002; McDonald et al., 2005). This bias is understandable from a conservation perspective but it is important to recognise and document species recovery especially where it could provide insights for improved conservation management. Interestingly, the expansion of *L. v. verreauxii* has occurred in the ongoing presence of Bd, even though Bd appears to cause some mortality. This suggests that changes in chytridiomycosis dynamics or the evolution of disease resistance or tolerance has increased the capacity of *L. v. verreauxii* to coexist with Bd. Our results demonstrate that some amphibian species are likely to recover, given sufficient time, from disease-driven declines and highlight the role of habitat in initial population persistence and subsequent re-expansion.

The pattern of local extirpation and subsequent re-expansion experienced by *L. v. verreauxii* contrasts sharply with that documented to date for many other Bd-susceptible amphibian species. For example, the emergence of chytridiomycosis has decimated upland central American amphibian assemblages with no sign of recovery (Lips et al., 2003). Likewise, in temperate Australia the critically endangered *P. corroboree* has experienced a prolonged decline with no evidence for population recovery (Hunter et al., 2010). However, initial observations suggest that some species may be starting to recover. In Venezuela, Rodriguez-Contreras et al. (2008) have speculated that lowland *Atelopus cruciger* population may be increasing, and in eastern Australia Richards and Alford (2005) and Newell et al. (2013) have documented increased density in *L. serrata* and *M. fleayi* populations, respectively, while McDonald (2002) and McDonald et al. (2005) have noted that the upper altitudinal limit of *L. serrata* appears to be increasing.

Lower host densities may lead to recovery of populations due to altered disease dynamics when declines are driven by density-dependent diseases (Briggs et al., 2010) and may have contributed to the re-expansion we document. On the Southern Highlands, the initial emergence of Bd was associated with the almost complete extirpation of four frog species, and greatly reduced distribution and abundance of *L. v. verreauxii* (the decline of *L. v. verreauxii* was confirmed by our baseline surveys) (Osborne, 1990, 1992; Osborne and Hunter, 1998; Osborne et al., 2008; Hamer et al., 2010). Decreases in population density of all frog species following the initial chytridiomycosis epidemic is likely to have substantially lowered rates of aquatic reinfection, reducing chytridiomycosis-mortality in remaining populations (Briggs et al., 2010). However, observations of diseased and dead frogs at sites with reasonable adult abundance, in addition to high infection burdens observed in some individuals are not entirely consistent with the scenario described by Briggs et al. (2010) and other factors are likely to be important. One potential explanation is that if a site survives the initial epidemic, reduced pathogen pressure decreases the rate of adult mortality to a level at which recruitment is sufficient or compensatory (Muths et al., 2011; Tobler et al., 2012). If individuals are uninfected at metamorphosis they are unlikely to contract Bd during their terrestrial juvenile phase (Hossack et al., 2013) and may survive to sexual maturity Bd-free. Under this scenario, populations could potentially increase in abundance despite high Bd prevalence in breeding adults if they are able to reproduce before succumbing to the disease.

Although changes in disease and population dynamics provide a plausible explanation, we cannot rule out the role of changes in host resistance, tolerance or decreased pathogen virulence (Altizer et al., 2003). The emergence of novel diseases in naïve populations can lead to rapid changes in host tolerance and resistance or pathogen virulence, resulting in greatly reduced mortality levels (Altizer et al., 2003). Savage and Zamudio (2011) have demonstrated the evolution of resistance to Bd and Woodhams et al. (2010) have suggested that variation in antimicrobial skin peptides among frog populations may have evolved in response to selection pressure from Bd. Variation in host tolerance

294 and/or resistance to Bd (Tobler and Schmidt, 2010) may provide an explanation for the re-expansion
295 of populations with endemic Bd infections. If infection is not inevitably lethal, then population
296 persistence with Bd is possible (Briggs et al., 2005). Concurrent experimental research on *L. v. alpina*
297 is investigating the potential for genetically based variation in susceptibility and will aid our
298 understanding of potential recovery mechanisms (S. Cashins 2013, James Cook University, personal
299 communication).

300

301 Site colonisation is likely related to distance from the nearest occupied site (Rannap et al., 2009; Hilje
302 and Aide, 2012), but data limitations prevented a thorough investigation of colonisation rates.
303 However, the colonisation of several sites in 2012 (Fig. 1) provides an insight on potential dispersal
304 distances. If the sites were colonised by individuals from the nearest known present site in 2011 the
305 dispersal distance would have been approximately two kilometres and would have involved
306 movement across undulating woodland. Because the distance between potential breeding habitats in
307 our study region is commonly less than two kilometres, we anticipate continued expansion.

308

309 Habitat suitability is also likely to influence successful site colonisation (Rannap et al., 2009). We
310 found that sites that were colonised were associated with higher levels of coarse woody debris (Table
311 1) which is likely to provide an important refuge for new colonisers (Hazell et al., 2003). Coarse
312 woody debris is a key resource for some amphibians (Indermaur and Schmidt, 2011) and its addition
313 could potentially be used to encourage *L. v. verreauxii* colonisation. Sites that supported frogs in both
314 survey periods were associated with lower levels of bare bank and higher levels of tussock in the
315 riparian zone (Table 1). These variables are generally associated with permanent water bodies and
316 may indicate minimal water level fluctuation during the breeding season (Hazell et al., 2003).
317 Because *L. v. verreauxii* attaches its eggs to vegetation below the water surface, substantial
318 fluctuations in water level can cause egg mortality (Hazell et al., 2001; Hazell et al., 2003). Sites with

increased water permanency may also be more resilient to the impacts of chytridiomycosis because they are buffered against mortality associated with recruitment failure in dry years. However, increased water permanency may also provide favourable conditions for Bd persistence (Kriger and Hero, 2007; Murray et al., 2011). Because environmental variables may influence population outcome following chytridiomycosis emergence (Murray et al., 2011; Savage et al., 2011), it is imperative to conserve species across their full range of habitats to increase their capacity to persist with novel threats, such as disease (Puschendorf et al., 2011). Considering biodiversity conservation more broadly, the lesson is that maintaining high quality habitat can be critical to species survival in the face of novel shocks such as pandemic diseases.

5. Conclusion

We demonstrate that *L. v. verreauxii* populations have begun to re-expand in the presence of Bd despite experiencing historical declines that were most likely caused by this pathogen. We anticipate that *L. v. verreauxii* will continue to expand across its former range. Because there is an urgent need to develop management actions for species that are experiencing ongoing declines (Woodhams et al., 2011), an exciting opportunity for solutions to this need lies in investigating Bd dynamics in recovering populations. This knowledge is likely to inform the development of effective response strategies (Tobler et al., 2012). In addition, from an immediate land-management point of view, our finding that habitat may influence population outcome and facilitate colonisation highlights the need to maintain high quality habitat across the entire historical range of a species. With potential additive impacts from future climate change, habitat loss and disease combining to create an increasingly pessimistic outlook for amphibians (Hof et al., 2011), our results provide optimism and hope for this imperilled taxa.

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512

513 Table 1. Model coefficients (and standard errors) of habitat variables from the best ranked model.
514 Recolonised sites were used as the baseline in the analysis so coefficients represent differences
515 relative to recolonised sites.

	Model terms			
Site type	(Intercept)	Bare bank	Tussock cover	Debris
Absent	1.49±0.41	0.82±0.53	-0.76±0.57	-0.55±0.22
Present	0.01±0.53	-1.33±1.14	0.49±0.62	-0.38±0.29

516

517 Table 2. Real-time PCR results for adult *L. v. verreauxii* (see Fig. 1 for sample locations). zse =
518 zoospore equivalents. Bd = *Batrachochytrium dendrobatidis*.

Site	Site type	No. samples	Bd prevalence	95% CI	Mean zse	Median zse
1	Colonised 2011	16	81	54-95	1299	358
2	Colonised 2011	17	88	62-98	23225	4264
3	Colonised 2011	12	67	35-89	2858	1047
4	Baseline present	20	80	56-93	8863	415
	Total	65	80	68-88	9267	704

519

520

Fig. 1. Location of study sites in south-eastern Australia. A. Baseline (1990 or 1996) present and absent sites are represented by open circles and crosses, respectively. Icons surrounded by large circles represent historical *L. v. verreauxii* sites from 1975-1976. B. Sites colonised during the study are represented by pluses for 2011 and bold pluses for 2012. Open squares represent sites colonised in 2011 but absent in 2012. Baseline present sites that were absent in 2011 and 2012 are represented by asterisk. Present and absent sites that did not change status during the study are represented by open circles and crosses, respectively. Bd sample locations are identified in B.