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Presence of the amphibian chytrid fungus *Batrachochytrium dendrobatidis* in threatened corroboree frog populations in the Australian Alps

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ABSTRACT: Since the early 1980s, the southern corroboree frog *Pseudophryne corroboree* and northern corroboree frog *P. pengilleyi* have been in a state of decline from their sub-alpine and high montane bog environments on the southern tablelands of New South Wales, Australia. To date, there has been no adequate explanation as to what is causing the decline of these species. We investigated the possibility that a pathogen associated with other recent frog declines in Australia, the amphibian chytrid fungus *Batrachochytrium dendrobatidis*, may have been implicated in the decline of the corroboree frogs. We used histology of toe material and real-time PCR of skin swabs to investigate the presence and infection rates with *B. dendrobatidis* in historic and extant populations of both corroboree frog species. Using histology, we did not detect any *B. dendrobatidis* infections in corroboree frog populations prior to their decline. However, using the same technique, high rates of infection were observed in populations of both species after the onset of substantial population declines. The real-time PCR screening of skin swabs identified high overall infection rates in extant populations of *P. corroboree* (between 44 and 59%), while significantly lower rates of infection were observed in low-altitude *P. pengilleyi* populations (14%). These results suggest that the initial and continued decline of the corroboree frogs may well be attributed to the emergence of *B. dendrobatidis* in populations of these species.

KEY WORDS: Amphibian declines · Corroboree frog · Amphibian chytrid fungus

INTRODUCTION

Over the past 50 yr, extinctions and declines of amphibian species worldwide have been occurring at an alarming rate (Stuart et al. 2004). Within Australia, 8 frog species have not been seen in the wild since their initial observed declines, and a further 32 species have declined to levels warranting listing as either vulnerable or endangered (Hero & Morrison 2004). These declines have primarily occurred along the eastern ranges of Australia, from the alpine regions in the southern highlands to the wet tropics rainforest regions of North Queensland (see reviews in Campbell 1999). While frog declines in North Queensland have primarily been documented for upland riverine species (McDonald & Alford 1999), in southeastern Australia both riverine and pond-breeding species have exhibited rapid population declines from both high and low altitudes (Gillespie & Hines 1999, Mahony 1999, Osborne et al. 1999). Of particular concern is that

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many of the declines have occurred rapidly from areas of relatively pristine habitat within national parks and other reserve systems (Alford & Richards 1999, Osborne et al. 1999).

Research into the causes of frog declines within Australia has identified number of potentially contributing factors, including introduced fish species (Gillespie 2001), habitat disturbance (Hero & Morrison 2004), increased drought frequency (Osborne 1989), increased UV-B radiation (Broomhall et al. 2000), the widespread use of agricultural chemicals (Hamer et al. 2004), and disease (Berger et al. 1998). The most substantial evidence for the cause of amphibian declines from relatively pristine upland environments is that they are the result of an outbreak of a disease known as chytridiomycosis, caused by infection with the amphibian chytrid fungus *Batrachochytrium dendrobatidis* (Berger et al. 1998, Skerratt et al. 2007). It is hypothesised that this pathogen was recently introduced into the Australian environment (Berger et al. 1998), possibly from South Africa (Daszak et al. 2003, Weldon et al. 2004), which may explain why some species appear to be particularly susceptible to this pathogen. Both genetic (Morehouse et al. 2003, Morgan et al. 2007) and predecline screening for infection (Berger et al. 1998) supports the novel pathogen hypothesis. Although retrospective data are considered by some to be insufficient to settle this issue (McCallum 2005, Rachowicz et al. 2005), a prospective study demonstrated that population declines of upland amphibians in Panama followed the arrival of *B. dendrobatidis*, supporting the epidemic wave hypothesis originally proposed on retrospective data (Lips et al. 2006, 2008).

Two closely related frog species that have undergone recent declines in southeastern Australia are the northern and southern corroboree frogs *Pseudophryne corroboree* and *P. pengilleyi*, respectively. Both these species occur in high montane and sub-alpine bog environments, where they typically breed in small ephemeral pools and seepage lines (Osborne 1989). Because the decline of these species initially coincided with a drought period during the early 1980s, it was suggested that drought, causing early pool drying and tadpole mortality, was the primary mechanism for the observed declines (Osborne 1989). However, despite subsequent periods of suitable climatic conditions, these species have continued declining across much of their range (Osborne et al. 1999). *P. pengilleyi* is currently listed as nationally vulnerable, while *P. corroboree* is listed nationally as endangered. If current population trends continue, *P. corroboree* is likely to become extinct in the wild within the next 5 to 10 yr (D. A. Hunter unpubl. data).

The present study was undertaken as an initial investigation into whether patterns of the emergence of infection with *Batrachochytrium dendrobatidis* in populations of both corroboree frog species is consistent with the possibility that this pathogen contributed to the initial and continued decline in these species. Histological techniques were used to assess the presence of *B. dendrobatidis* before and after population declines were first observed using preserved museum specimens and material collected from the field. Subsequently, skin swabs and real-time PCR was used to assess prevalence of infection in both species. Real-time PCR is a more sensitive test for *B. dendrobatidis* than histology in experimentally infected frogs (Boyle et al. 2004) and in wild frogs (Kriger et al. 2006) but was not available prior to 2004.

MATERIALS AND METHODS

Study species. The southern corroboree frog *Pseudophryne corroboree* and northern corroboree frog *P. pengilleyi* are closely related species that breed in high montane and sub-alpine bog environments in southeastern New South Wales, Australia. Male corroboree frogs call from terrestrial nest sites around the edge of ephemeral pools that are typically dry during the summer breeding period. Females lay their eggs in these nest sites, and the eggs develop through to a hatching stage and then enter diapause and await sufficient rains in autumn and winter to flood the nest site and allow the eggs to hatch the tadpoles to move through to the main pool. The tadpoles are then free swimming and feeding until metamorphosis (Anstis 2002).

Survey of archived specimens. Accession lists of archived specimens of both species held in the Australian National Wildlife Collection (ANWC) and the Australian National Museum (ANM) were obtained and a toe taken from all ANWC specimens and 35% of ANM specimens for histological examination. Archived frogs had been collected solely for the purpose of museum acquisition and availability for subsequent study and were presumed to be normal specimens.

Field survey. The locations of male nest sites were determined using the shout-response technique during the peak breeding period in mid-January, which involves shouting loudly at breeding habitat, to which corroboree frogs respond with their threat call (Osborne et al. 1999). The position of responding males is then determined using triangulation and marked with flagging tape for later inspection to locate the male. To avoid disturbing the males during the core breeding period, attempts to locate males were under-

taken towards the end of the breeding season in mid-February. Between 1997 and 2000 inclusive frogs were surveyed for chytridiomycosis using the histological examination of toe clips. After the PCR technique became available, frogs were sampled only by swabbing.

The procedure for obtaining toe material in the field involved removing a single toe at the base of the third phalange using a pair of dissecting scissors, and immediately preserving the toe in 70% ethanol. The swabbing procedure involved holding the frog by the back legs and wiping the frog 3 times on each of the feet, hands, inside and outside of the thighs, and stomach and back region. After the swab sample was taken, a digital photograph was also taken of the belly and throat pattern for each frog so as to check for recaptured individuals during future sampling. The swabs were stored in a cool location (below 10°C) until delivery to the Commonwealth Scientific and Industrial Research Organisation (CSIRO) Animal Health Laboratory in Geelong, Victoria.

Diagnostic techniques. ***Histology and screening of toe material:*** Preserved toe material from museum specimens collected prior to 1980 and toe material collected from extant populations between 1997 and 2000 were examined for infection with *Batrachochytrium dendrobatidis* using histology. The toes collected between 1997 and 2000 were decalcified in 15% formic acid for 20 h and then embedded vertically in paraffin wax. The toes obtained from museum specimens were decalcified in 10% formic acid for 48 h and then embedded horizontally in paraffin wax. The difference in the initial processing was due to the field-collected toes being originally processed for skeletochronology analysis. The toes were sectioned using a wax microtome to create ribbons of 5 µm sections. For the vertically embedded toes, 15 to 20 transversal sections were taken, while for the horizontally embedded toes 8 longitudinal sections were taken from the middle of the toe. The ribbons of sections were placed into a water bath mixed with 2% laboratory-grade gelatine, and then mounted on a microscope slide. After drying, sections were stained using the routine Mayer's haematoxylin and eosin (H&E) procedure. The slides were then mounted with a 60 mm cover-slip. The slides were assessed for *B. dendrobatidis* infection by visually scanning the areas of stratum corneum and stratum granulosum for each toe section using a light microscope set at 200× magnification, and then 400× magnification to confirm the presence of *B. dendrobatidis* zoosporangia (following recommended methods by Berger et al. 1999). All histological samples were assessed blind (i.e. no knowledge of collection date) by one of the authors (D. Mendez) who is highly skilled at identifying *B. dendrobatidis* infection using histological techniques.

PCR: The swabs were screened for the presence of *Batrachochytrium dendrobatidis* DNA using Taqman real-time PCR assay (see Boyle et al. [2004] and Hyatt et al. [2007] for details of this procedure). Inhibitors in samples were detected by use of a DNA amplicon (VICTM dye, Applied Biosystems) not found in nature and if this failed to amplify, it indicated that inhibitors of the PCR reaction were present in the sample (Hyatt et al. 2007). Inhibited samples were then diluted 1:10 or 1:100 and retested in an attempt to dilute the inhibitor below a critical threshold. If dilution was unsuccessful, the result was recorded as sample inhibited.

Statistical analysis. Uncertainty around the total proportion of adults testing positive for infection with *Batrachochytrium dendrobatidis* was estimated using a Bayesian approach with uninformative priors. The 95% credible intervals were propagated using Markov chain Monte Carlo methods with 100 000 samples after the first 10 000 samples were discarded. This was undertaken using the WinBUGS software package, version 1.4 (MRC and Imperial College of Science, Technology and Medicine) (Spiegelhalter et al. 2003).

Hygiene protocols. The following procedures were undertaken to minimise disease transmission between sites and between individuals within sites. Before entering the sites, all equipment that came into contact with frogs (both directly and indirectly) was sterilised with 90% ethanol. Each individual frog was handled using a new pair of disposable rubber gloves and a new plastic snap lock bag. Both items were immediately discarded after the frog was processed and a new set used for the next frog. Between processing individual frogs, scissors were sterilised using 90% ethanol.

RESULTS

All time periods were not evenly represented by the archived specimens, since there were no corroboree frog specimens collected between 1979 and 1991 available in the museums. The earliest museum specimen we sampled was collected in 1963. Of the 122 pre-1980 museum archived corroboree frog specimens sampled for infection with *Batrachochytrium dendrobatidis* using histology, no specimens tested positive for infection; whereas, of the 23 archived museum corroboree frogs we sampled that were collected between 1991 and 1993, 14 (61%) tested positive (Table 1). Of the 389 corroboree frog specimens collected from the field between 1997 and 2000 and sampled using histology, 14 tested positive for infection with *B. dendrobatidis* (Table 1). Because there was no overlap in the 95%

Table 1. *Pseudophryne corroboree* and *P. pengilleyi.* Results for the histological screening of individuals across populations before and after observed declines in these species. CI: credible interval; nd: no data

Period	No. of sites	No. of samples	No. positive	Proportion infected	95% CI
Pseudophryne corroboree					
Archived					
1960–1969	2	56	0	0	0–0.06
1970–1979	3	7	0	0	0–0.38
1980–1989	nd				
1990–1999	2	12	9	0.75	0.47–0.91
Field					
1998–2000	5	105	9	0.09	0.05–0.15
P. pengilleyi					
Archived					
1960–1969	3	48	0	0	0–0.07
1970–1979	2	11	0	0	0–0.27
1980–1989	nd				
1990–1999	3	11	5	0.45	0.21–0.69
Field					
1998–2000	12	284	5	0.02	0.01–0.04

Table 2. *Pseudophryne corroboree.* Real-time PCR results for amphibian chytrid fungus sampling undertaken across remnant *P. corroboree* populations in 2005 and 2006. Note: calculation for proportion positive and 95% credible intervals (95% CI) excluded inhibited samples

Site	No. of samples	No. inhibited	No. positive
2005			
Jagumba	4	0	3
Big Dargals 2	6	0	6
Far Dargals	10	0	3
Far Dargals 2	4	0	1
Upper Jagumba	2	0	1
Manjar	3	0	0
Far Manjar	1	0	0
Hell Hole	1	0	1
Ogilives Montane	3	0	0
Snakey	5	0	2
Upper Snakey	2	0	1
Total	41	0	18
Proportion positive (95% CI)			0.44 (0.30–0.59)
2006			
Upper Jagumba	8	3	4
Upper Snakey 2	1	0	1
Dargals Flat	4	2	1
Dargals Mountain	1	0	0
Dargals Saddle	4	0	3
Snakey	6	2	2
Upper Snakey	2	0	1
Far Manjar	1	0	0
Maragle	2	0	1
Far Dargals	8	0	5
Far Dargals 2	4	0	2
Hell Hole	4	1	2
Upper Ogilives	2	2	0
Total	47	10	22
Proportion positive (95% CI)			0.595 (0.43–0.74)

credible intervals, differences between pre-1980 and post-1980 infection rates were considered significant for the archived museum specimens (Table 1). Significant differences were also observed between the post-1980 archived specimens, and the specimens collected from the field between 1998 and 2000 (Table 1).

The overall levels of *Batrachochytrium dendrobatidis* infection determined by real-time PCR screening of skin swabs were very high across remnant population of *Pseudophryne corroboree* in both 2005 and 2006 (Table 2). Given the considerable overlap in 95% credible intervals, the differences in the proportion of individuals infected between 2005 and 2006 were not considered significant. Comparisons were not undertaken among populations because of the low number of individuals present/sampled in each population. The overall level of infection observed across *P. pengilleyi* populations in 2006 using real-time PCR screening was significantly lower than infection rates observed across *P. corroboree* populations during 2005 and 2006 (Tables 2 & 3). High levels of inhibition of the PCR assay were observed across samples in 2006, with the highest inhibition being observed in the *P. pengilleyi* populations (Tables 2 & 3). Inhibition was not observed in 2005 because samples were diluted until the influence of the inhibitors was negligible (Hyatt et al. 2007).

Table 3. *Pseudophryne pengilleyi.* Real-time PCR results for amphibian chytrid fungus sampling undertaken across *P. pengilleyi* populations in 2006. Note: calculation for proportion positive and 95% credible intervals (CI) excluded inhibited samples

Site	No. of samples	No. inhibited	No. positive
Bogong Peaks	11	0	2
Big Plain C	4	0	0
Big Plain C	4	1	0
Big Plain E	9	8	1
Big Plain A	7	3	0
Devils Peak	3	3	0
Brumby Flat	3	1	0
Pabral Rd.	2	2	0
Cooleman	15	7	0
Brindabella	15	10	1
Barnets Rd.	15	13	0
Broken Cart	15	14	0
Micalong Swamp	21	11	4
Notingham Rd.	14	11	0
Swamp Creek	11	11	0
Total	149	95	8
Proportion positive (95% CI)			0.14 (0.08–0.26)

DISCUSSION

Distribution and levels of infection in extant corroboree frog populations

The present study identified the presence of *Batrachochytrium dendrobatidis* across the distribution of both corroboree frog species (Fig. 1), with considerable variation in infection levels between the 2 species and among the different sampling periods (Tables 1, 2 & 3). An important feature of these results is the high level of infection observed in very small populations of *Pseudophryne corroboree* (Table 2). These results have several possible explanations. High infection at low densities may be expected if the pathogen is relatively benign to the host species (Anderson 1979). If *P. corroboree* is susceptible to *B. dendrobatidis*, then this level of infection could be expected if other factors are enhancing infection rates, such as the presence of non-susceptible reservoir host species (Gog et al. 2002, McCallum 2005). Confidently interpreting these results would require information on how infection

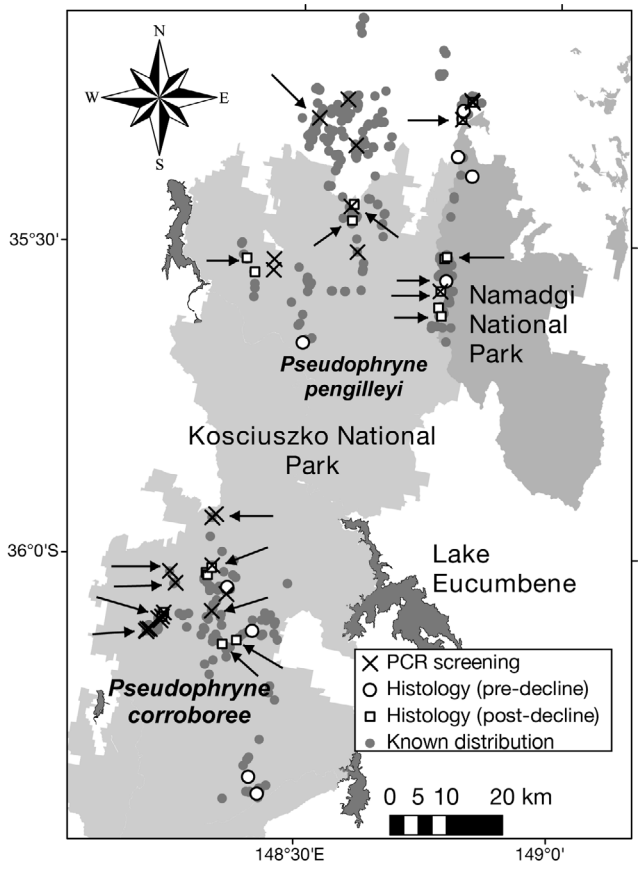

Fig. 1. Location of sites sampled for *Batrachochytrium dendrobatidis* infection in *Pseudophryne corroboree* and *P. pengilleyi* populations. Arrows point to sites where positive infection was recorded

with this pathogen influences *P. corroboree* survival in the field. *P. corroboree* certainly appears to be susceptible to this pathogen in captivity (G. Marantelli unpubl. data), however the response of corroboree frogs to infection with this pathogen in captivity may not be indicative of its response in the wild owing to environmental factors.

Another important feature of these results is the significantly lower levels of infection observed in extant *Pseudophryne pengilleyi* populations compared to extant *P. corroboree* populations (Tables 2 & 3). This is interesting because monitoring data suggests that many of these *P. pengilleyi* populations have not displayed population declines in recent years to the same extent as *P. corroboree* populations or *P. pengilleyi* populations at higher altitudes (Osborne et al. 1999). If *Batrachochytrium dendrobatidis* is involved in the continued decline of corroboree frogs, then the different population trajectories observed among different areas may be attributed to variation in rates of infection with *B. dendrobatidis*. While this result may be an artefact of sampling variation associated with fluctuations in detectable infection rates (Berger et al. 2004, Kriger & Hero 2006), this may also be a plausible hypothesis for a number of reasons. During the breeding season in summer, lower-altitude *P. pengilleyi* populations occupy a warmer and drier environment than *P. corroboree* populations or high-altitude *P. pengilleyi* populations (Osborne 1989), which are conditions less conducive to *B. dendrobatidis* spread and pathogenicity (Johnson & Speare 2003, Woodhams et al. 2003, Berger et al. 2004). The potential mechanisms for lower infection in low-altitude *P. pengilleyi* populations, or reduced pathogenicity, should be further investigated, as it may provide critical information about the ecology of *B. dendrobatidis* in corroboree frog populations.

While we observed significant differences in overall infection rates among the 3 main sampling periods when *Batrachochytrium dendrobatidis* was detected (Tables 1, 2 & 3), biological explanations for much of this are confounded by the different screening techniques we used among these periods (i.e. histology of archived specimens, histology of field material, PCR screening). PCR has been shown to be a more sensitive test for *B. dendrobatidis* than histology in experimentally infected frogs (Boyle et al. 2004, Hyatt et al. 2007) and in wild frogs (Kriger et al. 2006), and it is possible that the different histological techniques used between the field-collected and archived specimens may have also influenced detectability of infection. With regards to sampling procedures, an important aspect of the results of this study was the high proportion of real-time PCR samples expressing inhibition (Tables 2 & 3). Hyatt et al. (2007) suggested that one factor increasing the probability of inhibition was the presence of foreign material, such as dirt, on

the swabs. This may explain our results, because *Pseudophryne pengilleyi* swabs from populations where high rates of inhibition where observed typically had greater quantities of dirt on them as a result of the frogs at these sites more often occupying partially earthen nest sites, as opposed to the vegetation nest sites at other sites (D. A. Hunter pers. obs.). While repeated dilutions of inhibited samples may reduce the influence of these inhibitors, as was undertaken for the 2005 *P. corroboree* samples, this procedure may increase the rate of false negative results (A. Hyatt pers. comm.), and also increases the cost per sample.

Presence of the amphibian chytrid fungus pre- and post-decline

The present study did not detect the presence of *Batrachochytrium dendrobatidis* in corroboree frog populations prior to observed declines in these species, which began in the early 1980s (Osborne 1989). This is consistent with the results of other Australian studies that have undertaken retrospective screening of preserved frog specimens (Berger et al. 1998, Aplin & Kirkpatrick 2000), with the earliest record of *B. dendrobatidis* in Australia being 1978 (Berger et al. 1998). Berger et al. (1998) outlined several possible explanations for not detecting *B. dendrobatidis* in frog populations prior to observed declines, including that this pathogen only recently spread through the Australian environment. Determining whether *B. dendrobatidis* is a novel pathogen in Australia is fundamental to understanding and responding to frog declines attributed to this pathogen, because the hypothesis that it is endemic and only recently attained increased virulence implies that other factors are likely to be involved and need to be identified. While genetic data (Morehouse et al. 2003, Morgan et al. 2007) and pre-decline screening for infection support the novel pathogen hypothesis, this evidence was not considered sufficiently robust to adequately address this hypothesis by some authors (McCallum 2005). The prospective study by Lips et al. (2006) has weakened arguments against chytridiomycosis not manifesting as an epidemic wave in chytrid-free areas (Skerratt et al. 2007).

As was pointed out by McCallum (2005), the strength of evidence of retrospective screening is limited in supporting the novel pathogen hypothesis. The level of sampling undertaken in the present study was only statistically confident in detecting the presence of *Batrachochytrium dendrobatidis* if the levels of detectable infection were greater than 0.06%, which overlaps with the 95% credible intervals for the post-decline infection rates we detected using histology (Table 1). Hence, we are limited in suggesting that *B. dendrobatidis* was not present in the environment prior to these declines. Interpreting pre- and post-decline comparisons is confounded by a range of unknown factors, including the potential for infection to vary greatly between both seasons and years (Berger et al. 2004), and the potential for high rates of false negatives because of the limited sensitivity of histological screening for infection (Kriger et al. 2006). Despite the limitations for supporting the novel pathogen hypothesis, retrospective screening is a very powerful technique for rejecting this hypothesis, as only one positive sample prior to observed declines is required. Hence, retrospective screening should continue to be undertaken as a means to furthering our understanding about the emergence of *B. dendrobatidis* in frog populations.

Disease hypothesis and the decline of the corroboree frogs

The pattern of decline of corroboree frogs has similarities with the decline of other frog species for which *Batrachochytrium dendrobatidis* has been suggested as the primary causal agent. The initial decline observed for the corroboree frogs involved a reduction in the breeding adult population size that would have required an increase in adult mortality, not just failed recruitment to metamorphosis (Hunter 2000). Hence hypotheses implicating factors causing mortality during the post-metamorphic stages are more parsimonious than hypotheses involving only failed recruitment to metamorphosis (Scherer et al. 2005). In addition to this, the apparent altitudinal relationship in the observed corroboree frog declines, in combination with the fact that the high-altitude populations are in cooler and moister habitats, is also consistent with the pattern of decline observed in other frog species where *B. dendrobatidis* has been implicated (Berger et al. 1998, McDonald et al. 2005).

Unlike other studies where *Batrachochytrium dendrobatidis* has been associated with declining frog populations (Berger et al. 1998, Lips 1999, Lips et al. 2006), sick and/or dead frogs infected with *B. dendrobatidis* have not been located during the monitoring of declining corroboree frog populations. Even if moribund frogs were present in declining corroboree frog populations, locating these individuals may not be expected because these species are typically concealed within vegetation, and sick individuals are unlikely to respond to the survey technique (shout-response). The fact that this study sampled apparently healthy individuals reduces at least one form of sampling bias associated with comparing infection levels between different periods, areas, or species (McCallum 2005).

There remains some conjecture over the origins of *Batrachochytrium dendrobatidis* and its role in the decline of frog species along the eastern ranges of Australia (McCallum 2005, Rachowicz et al. 2005). It may not be possible to adequately determine the role of this pathogen in the initial decline of corroboree frogs, because sampling during the early 1980s was not undertaken. Both corroboree frog species now have endemic chytridiomycosis, a situation similar to that which occurred after the epidemic wave in rainforest frogs in central and north Queensland (Retallick et al. 2004, McDonald et al. 2005). Species in central and north Queensland that did not decline rapidly to extinction (Schloegel et al. 2006) appear now to have stabilised and represent an epidemiological pattern of initial declines, partial recovery, and stability. This pattern may be occurring for low-altitude populations of *Pseudophryne pengilleyi*. However, we hypothesise that *P. corroboree* and high-altitude populations of *P. pengilleyi* are showing a different pattern with a progressive decline, initially rapid, but now at a slower rate (Osborne et al. 1999). This epidemiological pattern will lead to extinction unless the cause can be addressed.

Hence, it is important that research is undertaken to determine whether *Batrachochytrium dendrobatidis* is contributing to the continued decline of these species and if it is, to take measures to counteract it. Since other frog species in the Australian Alps region underwent similar rapid population declines starting in the early 1980s (Osborne et al. 1999), a coordinated approach to assessing the influence *B. dendrobatidis* on declining alpine frog species would be a more appropriate context. This would not only benefit the implementation of the corroboree frog recovery program, but would make an important contribution to assessing the broader implication of *B. dendrobatidis* to recent frog declines along the eastern ranges of Australia.

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