Vol. 100: 191–200, 2012
doi: 10.3354/dao02494

DISEASES OF AQUATIC ORGANISMS
Dis Aquat Org

Published September 12

Prevalence of *Batrachochytrium dendrobatidis* infection is extremely low in direct-developing Australian microhylids

Kim F. Hauselberger*, Ross A. Alford

School of Marine and Tropical Biology, James Cook University, Townsville, Queensland 4811, Australia

ABSTRACT: The emerging infectious disease chytridiomycosis has been implicated in declines and disappearances of amphibian populations around the world. However, susceptibility to infection and the extent of pathological effects of infection vary among hosts, and species with life histories that include parental care of direct-developing terrestrial eggs may tend to be less susceptible. We examined samples from a total of 595 individuals of 9 species of direct-developing Australian frogs in the family Microhylidae for the presence of infection by *Batrachochytrium dendrobatidis* (*Bd*). Between 1995 and 2004, 336 samples were collected; 102 of these were analysed histologically and 234 were tissues stored in alcohol, which were examined using diagnostic quantitative PCR (qPCR). Swab samples were collected from 259 frogs from 2005 to 2008 and were examined using qPCR. None of the 595 samples showed evidence of infection by *Bd*. If these data are regarded as a single sample representative of Australian microhylids, the upper 95% binomial confidence limit for the prevalence of infection in frogs of this family is 0.0062 (<1%). Even if only the data from the more powerful diagnostic qPCR tests are used, the upper 95% confidence limit for prevalence is 0.0075 (<1%). Our data suggest that Australian microhylids have a very low prevalence of infection by *Bd* in nature, and thus are either not susceptible, or are only slightly susceptible, to chytridiomycosis. This could be due solely to, or in combination with, low rates of transmission and to factors that promote resistance to infection, including ecological or behavioural characteristics, innate immune functions such as antimicrobial skin peptides, or antimicrobial symbionts in skin flora.

KEY WORDS: Chytridiomycosis · Microhylidae · *Cophixalus* · *Austrochaperina* · qPCR

INTRODUCTION

Pathogenic organisms are frequently cited as important drivers of community dynamics (Hudson et al. 2001), and disease-causing organisms have been implicated in declines and extinctions of a wide variety of wildlife species (Daszak et al. 1999). In these scenarios, some species experience severe declines, others decline less severely, and others suffer no losses. We use susceptibility to a disease to mean the propensity to become infected by the causative pathogen and develop symptoms that produce morbidity or mortality; this can vary greatly among and even within species (Wakelin 1978). We use tolerance of infection to mean the ability to carry an infection by a pathogen with minimal development of symptoms and little or no morbidity or mortality (e.g. Puschendorf et al. 2011). Resistance is the inverse of susceptibility, and we suggest that susceptibility to disease can be thought of as the product of several factors: rates of transmission, level of tolerance of infection, and rates of clearance (loss) of established

*Email: kim.hauselberger@my.jcu.edu.au

infections. Disease dynamics are affected by susceptibility, which in turn is affected by a variety of factors related to the host, the pathogen, and the surrounding environment (Warner 1968, Van Riper et al. 1986, Lips et al. 2006).

Chytridiomycosis is an emerging infectious disease caused by *Batrachochytrium dendrobatidis* (*Bd*), a chytrid fungus that parasitises the mouthparts of larvae and the keratinised epidermis of postmetamorphic amphibians (Berger et al. 1998, Longcore et al. 1999). This pathogen has been associated with population declines of amphibians in many regions (Daszak et al. 2003, Lips et al. 2006, Skerratt et al. 2007), including at least 14 threatened Australian species (Department of the Environment and Heritage 2006). Chytridiomycosis causes mortality in a wide range of amphibians; however, susceptibility to it varies greatly among host species (Davidson et al. 2003, Stockwell et al. 2010) and among populations within species (Berger et al. 2005, Woodhams & Alford 2005). Where outbreaks of chytridiomycosis have occurred, it is common for some species to suffer local extinction, while other species decline and some suffer no population-level effects (McDonald & Alford 1999, Retallick et al. 2004, Lips et al. 2006, Schloegel et al. 2006).

Variation in susceptibility to chytridiomycosis can be associated with ecological and life-history traits of host species (Lips et al. 2003, Retallick et al. 2004, Woodhams & Alford 2005). The fungus requires a moist environment to multiply and release infective zoospores (Pessier et al. 1999), and has a relatively low maximum thermal tolerance (Piotrowski et al. 2004). The habitat or microhabitat used by species can affect rates of transmission of *Bd* (Rowley & Alford 2007). After individuals become infected, the thermal and hydric environments they experience can have a large influence on tolerance of infections (Woodhams et al. 2003, Bustamante et al. 2010, Murphy et al. 2011). Species that exist in environments that limit the growth of the fungus can have high tolerances of infection, leading to low susceptibility to the effects of chytridiomycosis even when the prevalence of infection is high (Puschendorf et al. 2011).

In Australia, frogs of species with life histories incorporating direct development (which therefore have no aquatic tadpole stage) have seldom been found infected by *Bd*. That alone provides little information about their susceptibility to chytridiomycosis, since low prevalences of infection can be caused by low rates of transmission, high rates of clearance of infections, or high rates of mortality of infected individuals that remove them from sampled populations (Puschendorf et al. 2011).

There are relatively few examples of population crashes of terrestrial, direct-developing species associated with *Bd.* They have occurred in Central America (Lips et al. 2006, Brem & Lips 2008), Puerto Rico (Burrowes et al. 2004, Longo & Burrowes 2010), and New Zealand (Bell et al. 2004). However, in general, terrestrial frogs with direct development have been more likely to survive epidemic outbreaks of *Bd* than have other tropical amphibians (Lips et al. 2003, Lips et al. 2006). Australian microhylids are not known to have suffered any population declines in the Wet Tropics (WT) region of Queensland, even when sympatric species declined during outbreaks of chytridiomycosis (McDonald & Alford 1999, Hauselberger & Alford 2005). This indicates that the disease has not been removing many individuals from populations, and thus that their susceptibility to chytridiomycosis may be low.

If direct-developing species are generally less susceptible to chytridiomycosis, this could be caused by lower rates of transmission because they are relatively unlikely to be exposed to the fungus' aquatic zoospores (Daszak et al. 1999). They lack tadpoles, which may become infected and retain infections as postmetamorphic frogs. They often call and spawn well away from water (Felton et al. 2006), which could reduce rates of interspecific transmission from species that breed aquatically. They can be highly philopatric (Hauselberger & Alford 2005, Felton et al. 2006), which could reduce intraspecific transmission rates. They could also have higher rates of clearance of infections because of more effective innate immune defences. Direct-developing species commonly exhibit some form of parental care of the egg clutch, which may prevent fungal infestation of the developing embryos (Forester 1979, Simon 1983). Egg-brooding species may also possess antimicrobial skin secretions or symbiotic bacterial flora that inhibit the growth of a range of bacteria and fungi, including *Bd* (Harris et al. 2006).

Previous research on the distribution of *Bd* in Australia and its effects on frogs has focused on taxa recognised as being threatened by declines. The pathogen has been found on at least 60 Australian species from 4 families (Hylidae, Microhylidae, Myobatrachidae, and Bufonidae; Murray et al. 2010). No declines related to chytridiomycosis have been reported in Australian species of the native families Microhylidae and Ranidae.

Most Australian microhylid species occur in the WT region (Cogger 1996), where extensive declines

have occurred in other species and *Bd* is now endemic (Berger et al. 2004, McDonald et al. 2005, Woodhams & Alford 2005). Although they do not breed aquatically, microhylids inhabit cool, moist habitats that should favour *Bd* growth and reproduction. Only 2 microhylid species (*Austrochaperina robusta* and *Cophixalus ornatus*), from 2 separate populations within the WT (Paluma and Babinda), have been surveyed for infection by *Bd*. Histological surveys of *A. robusta* at Paluma failed to detect any infected animals (Hauselberger 2001; D. Mendez pers. comm.), whilst a single swab sample collected from a *C. ornatus* that was located 2 m from a creek tested positive for *Bd* DNA using a diagnostic quantitative PCR (qPCR) assay (Kriger & Hero 2006). This sample was tested in triplicate (K. Kriger pers. comm.), and established that it is possible for microhylids to be infected by *Bd*. However, this single result did not provide information about the prevalence of *Bd* on microhylids or its distribution among species or populations. Exposure trials indicate that *C. ornatus* readily become infected when exposed to high concentrations of *Bd* zoospores in the laboratory, but are tolerant of infections, experiencing no mortality in a laboratory experiment, and clear infections relatively rapidly (Hauselberger 2011).

We designed the present study to determine prevalence of *Bd* infections in Australian microhylids in nature and to examine their distributions geographically and among taxa. This information is needed to understand the extent to which microhylids may be threatened by chytridiomycosis. If the prevalence of *Bd* infections is high in Australian microhylids, it is important to determine whether the cause of their apparent resistance to declines associated with chytridiomycosis is innate or is due to environmental factors. If environmental factors play a role, small changes in environmental conditions could greatly affect the prevalence and virulence of the pathogen (Woodhams et al. 2003, Pounds et al. 2006, Richards-Zawacki 2010). Because several species have very restricted geographic ranges (Williams et al. 2006), any change in environmental factors could affect those entire species over very short periods of time, and could lead to rapid extinctions caused by chytridiomycosis. If *Bd* occurs only at low prevalences or is absent from many populations, it may not constitute a great threat. However, if innate mechanisms are responsible for a high degree of resistance to infection, understanding these mechanisms may aid in captive management and treatment of frogs belonging to taxa that are more vulnerable to the pathogen.

MATERIALS AND METHODS

Diagnosis of *Bd* infection

A total of 493 samples were analysed using the diagnostic qPCR assay developed by Boyle et al. (2004); 451 of these were analysed at James Cook University (JCU), Townsville, and 42 were analysed at the Commonwealth Scientific and Industrial Research Organisation (CSIRO) Australian Animal Health Laboratory (AAHL), Geelong. Diagnostic qPCR is a much more sensitive assay than histology and is highly specific (Kriger et al. 2006, Hyatt et al. 2007). The protocol used in both the JCU and AAHL CSIRO PCR laboratories includes running each sample in triplicate and the use of an Applied Biosystem TaqMan® Exogenous Internal Positive Control that should reveal the presence of inhibition in samples (Hyatt et al. 2007). A total of 102 archived samples that could not be analysed using qPCR (see next subsection) were examined for the presence of *Bd* infection using histology (Berger et al. 2000).

Archived samples

A total of 336 archived samples were available from previous studies on microhylids of 7 species (*Cophixalus aenigma*, *C. bombiens*, *C. hosmeri*, *C. infacetus*, *C. neglectus*, *C. ornatus*, and *Austrochaperina robusta*). The samples consisted of toe clips and tissue sections preserved in 70% ethanol, or prepared slide sections of toe clips, stored at JCU and the University of Queensland. They were collected between 1995 and 2004 during 5 separate research studies (Brooke et al. 2000, Hauselberger & Alford 2005, Felton et al. 2006, Williams et al. 2006; C. Hoskin pers. comm.; Table 1). These studies followed collection and hygiene protocols similar to those used in the present study, with the exception that in some cases, before the need to prevent transmission of chytridiomycosis was fully appreciated, animals were handled with bare hands. This could potentially have led to cross-contamination of samples used in diagnostic PCR with *Bd* DNA, which could have inflated apparent prevalences. The nature of our results renders this possibility irrelevant (see Table 2).

A total of 234 ethanol-preserved tissue samples were processed for the detection of *Bd* DNA using qPCR. These included 201 toe clips collected by Brooke et al. (2000) from August 1995 to March 1996,

Table 1. Locations of archived (A) and swab (Sw) sample collection sites within the Wet Tropics region of northern Queensland

Sample type	Site	Location	Latitude S	Longitude E	Elevation (m)
A and Sw	AU2	Mena Creek	17° 40.01′	145° 52.60′	200
A and Sw	AU4	Henrietta Creek	17° 36.92′	145° 45.32′	400
A and Sw	AU6	South Johnston	17° 40.09′	145° 43.25′	600
Sw	AU10	Charmillin Creek	17° 40.86′	145° 31.19′	1000
A	BF1	Mt. Bartle Frere	17° 26.11′	145° 51.29′	100
A	BF7	Mt. Bartle Frere	17° 24.98′	145° 50.12′	700
A	BF13	Mt. Bartle Frere	17° 24.36′	145° 49.31′	1350
A and Sw	BK	Mt. Bellenden Ker	17° 15.84′	145° 51.31′	1550
Sw	CU8	Mt. Lewis	16° 35.38′	145° 17.35′	800
Sw	CU10	Mt. Lewis	16° 35.21′	145° 16.36′	1000
Sw	CU12	Mt. Lewis	16° 30.56′	145° 16.35′	1200
A	Hai	Mt. Haig	17° 05.60′	146° 36.09′	900
A	Mau	Mausman	17° 20.79′	145° 41.67′	740
A and Sw	Pal	Paluma	19° 00.42′	146° 12.43′	900

18 toe clips collected by Williams et al. (2006) from September 2003 to March 2004, and 15 tissue samples collected by J. Milton and C. Hoskin (see Table 2). Tissue samples consisted of a strip of skin cut from the ventral thigh or abdominal region. Each sample was removed from its storage vial using forceps and transferred to an individually labeled Eppendorf tube containing 50% ethanol. Forceps were sterilised by submerging in 100% ethanol and flaming between each use.

A total of 102 samples existed as prepared histological slides originally used for skeletochronological determination of age. Felton et al. (2006) collected toe clips from 47 *Cophixalus ornatus* at Paluma from 1998 to 1999, and Hauselberger & Alford (2005) collected toe clips from 55 *Austrochaperina robusta* in 2000 and 2001 at Paluma (see Table 2). These samples had been prepared as 7 µm sections, stained in haematoxylin and eosin, and mounted on slides. The slides were examined using a light microscope to detect *Bd* infection by searching for fungal structures in the stratum corneum, as recommended by Berger et al. (2000). The only departure from the technique of Berger et al. (2000) is that they recommend using 5 µm sections; this should not have affected diagnostic efficiency.

Swab samples

A total of 259 individuals belonging to 7 microhylid species (*Austrochaperina fryi*, *A. pluvialis*, *A. robusta*, *Cophixalus aenigma*, *C. hosmeri*, *C. neglectus*, and *C. ornatus*) were sampled for the presence of *Bd* infection by swabbing their skins. Nine 500 m transects were set up within the WT region (Table 1), and each transect was sampled at least once each wet season (September to March) from 2004 to 2008. Frogs were located and swabbed during visual encounter surveys during the day by rolling logs, and after being located by their vocalisations at night. Surface temperatures of frogs were measured using an infared (IR) thermometer (Raytek ST80 Pro-Plus Non-contact laser thermometer) before capture.

Frogs were captured in individual plastic bags, and strict hygiene protocols for handling frogs and equipment was followed (Speare et al. 2004). Frogs were swabbed using standard protocols (Hyatt et al. 2007) that produce the highest sensitivity using the qPCR assay to detect *Bd* DNA. To prevent contamination and reduction in the number of zoospores detected by the qPCR assay, swabs were stored at <5°C within 2 h of collection, or stored on ice and refrigerated as soon as possible after collection (Van Sluys et al. 2008).

Batching of samples

Due to the cost of qPCR analysis, some samples were analysed in batches. One hundred archived samples collected by Brooke et al. (2000) were analysed in batches of 10, where 10 toe clips were placed into a single container for qPCR analysis. This was only carried out on samples that contained more than one toe clip, so that if a positive result occurred, individual samples could be retested (Hyatt et al. 2007). Eighty swab samples collected from 2004 to 2008 were run in batches of 4, where 4 swabs were prepared together for qPCR analysis. All field samples collected consisted of 2 swabs per individual, so that if a positive result was found in the qPCR analysis, the second set of swabs could be individually retested.

Statistical analysis

We calculated 2-tailed Clopper-Pearson 95% binomial confidence limits for the prevalence of *Bd* in a variety of populations, using the program StatXact 4.0 (Cytel Statistical Software). Sample sizes and 2-

tailed Clopper-Pearson 95% binomial confidence limits were calculated separately for swab samples and for archived samples for each area surveyed. Confidence limits for swab samples were calculated using results for all samples taken at each site during the period 2004 to 2008. Confidence limits for archived samples were calculated independently for each location. Our confidence intervals assume that the diagnostic technique is 100% sensitive and specific. Unfortunately, no published data are available for the true sensitivity and specificity of any technique for diagnosing *Bd* infection in Australian microhylid frogs. Given the nature of our results, it is not likely that our conclusions would be strongly affected by the incorporation of such information.

RESULTS

Archived samples

A total of 336 archived samples, taken from 7 species, were examined for the presence of *Bd* using either histology (102 samples) or diagnostic qPCR (234 samples). All samples were negative (Table 2). This included groups of *Cophixalus neglectus* and *C. ornatus* individuals that were collected from Mt. Bellenden Ker in early 2001 and died in the laboratory shortly thereafter. It had been assumed that these individuals succumbed to chytridiomycosis; however, diagnostic qPCR analysis of tissue samples did not indicate that *Bd* DNA was present. The small sizes of many samples have produced high upper 95% confidence limits for prevalence (Table 2), but 2 samples examined histologically had upper 95% confidence limits below 10%, and the sample for Paluma in 1996, which was analysed using the more sensitive qPCR assay, had an upper 95% confidence limit of 1.8%. Aggregated across all archived samples, the upper 95% confidence limit for prevalence was 1.1%.

Table 2. *Batrachochytrium dendrobatidis* (*Bd*) infecting *Austrochaperina robusta* and *Cophixalus* spp. Upper 95% binomial confidence limits (CL) for the prevalence of *Bd* infection derived from archived samples using diagnostic quantitative PCR (qPCR) of tissue samples (T), qPCR of skin swabs (Sw), and histological analysis of toe clips (H). All samples returned negative results. Prevalences with upper 95% confidence limits of <10% are highlighted in **bold**. See Table 1 for site locations

Species	Date	Site	Analysis	n	Upper 95% CL
A. robusta	Oct 2000–Mar 2001	Pal	H	55	**0.065**
C. aenigma	Sep 2003–Mar 2004	AU6	T	4	0.602
C. bombiens	Sep 2003–Mar 2004	AU4	T	1	0.975
C. hosmeri	Sep 2003–Mar 2004	AU2	T	3	0.708
C. infacetus	Sep 2003–Mar 2004	AU4	T	6	0.459
C. neglectus	Jan 2001	BF13	Sw	1	0.975
	Sep 2003–Mar 2004	BK	T	4	0.602
C. ornatus	Sep 1995–Mar 1996	Pal	T	201	**0.018**
	Oct 1998–Mar 1999	Pal	H	47	**0.075**
	Jan 2001	BF1	Sw	4	0.602
	Jan 2001	BF7	Sw	4	0.602
	Jan 2001	BF13	Sw	4	0.602
	Jan 2001	Hai	Sw	1	0.975
	Jan 2001	Mau	Sw	1	0.975
Total				336	**0.011**

Field data

None of the 259 swab samples taken from 7 species during surveys from 2004 to 08 tested positive for the presence of *Bd* using diagnostic qPCR (Table 3). The small sample sizes at many sites produced high upper 95% confidence limits for prevalence, but at the 3 sites with larger samples, they were below 10%. Aggregating all the swab samples taken from 2004 to 2008 to estimate an upper 95% confidence limit for microhylid frogs in the Australian WT produced an upper 95% binomial confidence limit for prevalence of 1.4%. The surface temperatures of 237 of the frogs from which samples were collected from 2004 to 2008 ranged from 10.8 to 24.3°C (Table 4; the mean surface temperature was 18.1°C).

If all of the data for diagnostic qPCR assays of archived and survey samples (0 positives from 493 individuals) are combined as a sample of WT microhylids in general, the upper 95% binomial confidence limit for prevalence in this sample is 0.75%. The upper 95% confidence limit for the 102 samples examined histologically (all found to be negative) is 3.55%. Considering the total of 595 negative samples together gives an upper 95% binomial confidence limit of 0.62% for prevalence.

DISCUSSION

None of the samples analysed by diagnostic qPCR in the present study tested positive for the presence of *Bd* DNA. All diagnostic qPCR analyses were carried out under standardised conditions by laboratories that have helped to establish the standards for this technique and

Table 3. *Batrachochytrium dendrobatidis* (*Bd*) infecting *Austrochaperina* spp. and *Cophixalus* spp. Upper 95% binomial confidence limits (CL; final row) for population prevalence of *Bd* in field sites (2004–2008), using diagnostic quantitative PCR of skin swab samples. Values indicate number of frogs sampled, and all samples were negative for the presence of *Bd*. Prevalences with upper 95% CL of <10% are highlighted in **bold**. See Table 1 for site locations

Species	AU2	AU4	AU6	AU10	CU8	CU10	CU12	Pal	BK	Total
A. fryi					5	2				7
A. pluvialis	1				3					4
A. robusta		2	1	1				22		26
C. aenigma					1	2				3
C. hosmeri	2									2
C. neglectus									132	132
C. ornatus				57	2		1	23	2	85
Total	3	2	1	58	11	4	1	45	134	259
Upper 95% CL	0.71	0.84	0.98	**0.06**	0.28	0.60	0.98	**0.08**	**0.03**	**0.014**

Table 4. *Austrochaperina* spp. and *Cophixalus* spp. Maximum, minimum, and mean surface temperatures of frogs collected during the 2004–2008 field season. See Table 1 for site locations

Site	n	Skin temp. (°C)		
		Mean	Min.	Max.
AU2	3	22.8	21.2	24.3
AU4	2	22	21.9	22.1
AU6	1	20.4	20.4	20.4
AU10	56	19.8	17.1	23.8
CU8	11	21.2	20.6	21.9
CU10	4	19.5	19.2	19.6
CU12	1	18.4	18.4	18.4
Pal	41	21.2	19.2	24
BK	118	15.7	10.8	20.4
Overall	237	18.1	17.1	24.3

included all appropriate controls; false negative rates should thus have been very low. Considering only the results of the diagnostic qPCR of swab samples, at 2 sites (Bellenden Ker and Paluma) sample sizes were large enough to indicate with 95% confidence that the prevalence of *Bd* infection is below 5% and 10%, respectively. If *Bd* infections occur in these populations, they occur at prevalences well below those documented (even using histology, and using qPCR analyses by the same laboratories) for stream-associated frogs in the Australian WT (Berger et al. 2004, Woodhams & Alford 2005, Puschendorf et al. 2011), and also those found in neotropical direct-developing species (Longo & Burrowes 2010).

Aggregating samples taken across multiple species and sites is potentially problematic when examining the prevalence of infection. Mean prevalence can vary among species (Woodhams et al. 2006). A mean estimated from an aggregated sample including species with different means will thus be influenced by the exact composition of the sample, and including species that are not susceptible to infection could artificially reduce the estimated mean. However, we know that *Cophixalus ornatus*, which accounts for well over half of our samples, is susceptible to infection (Kriger & Hero 2006, Hauselberger 2011), and we have no reason to believe that other Australian microhylids are not. Similar aggregation has been done repeatedly in the literature on chytridiomycosis and is accepted as a reasonable approximation of overall prevalences and distributions of *Bd* infection (Weldon et al. 2004, Sanchez et al. 2008, Cheng et al. 2011). The results for our swab samples indicate that the upper 95% confidence limit for the prevalence of *Bd* infection aggregated across species and populations is 1.4%. Including the results of qPCR tests on alcohol-preserved samples raises the total to 493 individuals that returned negative qPCR results, and reduces the upper 95% confidence limit to <1%. Including the demonstrably less powerful histological results for the total of 595 negative individuals reduces it further. If we use only the 300 negative qPCR results for *C. ornatus*, which is known to be susceptible to infection by *Bd*, the upper 95% confidence limit for prevalence is 1.2%. However they are looked at, our data indicate that the prevalence of *Bd* in microhylids is very low in the Australian WT. This indicates that Australian microhylids have some combination of low susceptibility to infection by the pathogen, low rates of transmission, and high rates of clearance of infections.

It is possible that our results may include false negatives. The use of histological analysis instead of diagnostic PCR, low infection intensity, degradation of fungal DNA due to inhibitors or temperature, and batching samples can all lead to reduced sensitivity of

diagnostic assays. Hyatt et al. (2007) demonstrated that diagnosis of *Bd* infection via histology is less sensitive than qPCR in all but the very early stages of infection, but that qPCR assays of toe clips have a sensitivity similar to assays of swab samples. All the archived samples we analysed using qPCR were fixed and stored only in ethanol, which is not known to degrade the sensitivity of qPCR tests (Soto-Azat et al. 2009). Sample storage in ethanol is used as standard procedure by some laboratories (Hyatt et al. 2007). It is thus unlikely that the use of archived toe-clip samples in the present study produced false negatives.

Inhibitors can be present in a swab sample if foreign material such as dirt or detritus is picked up on the swab, and can also be present in skin secretions of some frog species or life stages (Hyatt et al. 2007). However, standard protocol in both the JCU and AAHL/CSIRO laboratories includes a positive control in each PCR run that should reveal the presence of inhibition, and no evidence of inhibition was ever detected in our samples. As it was not always possible to refrigerate samples collected in remote areas, increased temperatures could have led to a reduction of the amount of *Bd* DNA detected on swabs using the qPCR assay (Van Sluys et al. 2008). This effect was minimised, as air temperatures at sampling sites were typically <30°C, and swabs were always refrigerated or stored on ice <2 h after the samples were taken. Additionally, Hyatt et al. (2007) demonstrated that storing swabs at 23°C in the laboratory for 18 mo did not reduce detection sensitivity.

One aspect of our methods could have reduced the sensitivity of the qPCR assay for 100 of our archived samples. This was our decision to batch those samples into 10 groups of 10. Hyatt et al. (2007) showed that the maximum number of swabs that can be pooled without lowering the sensitivity of the qPCR assay is 5. Batching groups of >5 lowered the sensitivity of the test in their laboratory trials, especially for samples that contained a low number of *Bd* zoospores. Batching samples in groups of 10 produced false negative results in samples that were spiked with 1 and 10 zoospores, but did not produce negative results in samples that contained 100 zoospores (Hyatt et al. 2007). In the present study, batch samples that were run in groups of 10 were whole toe-clip samples rather than skin swabs. As heavier infections of *Bd* are concentrated on the legs and feet in at least some amphibian species (North & Alford 2008), and because whole tissue samples hold more *Bd* DNA than swabs, and may actually be preferable to swabs for detecting low-level infections (Longo et al. 2010), it is unlikely that batching produced more than a small number of false negatives, if it produced any. The pooling procedure was only used for half of the Paluma collection of archived samples. The other half of those samples were analysed individually and also returned uniformly negative results.

Although it is impossible to state with 100% certainty that no false negatives occurred in some of the 493 diagnostic qPCR results, they are unlikely to have affected a high proportion of the samples. Because diagnosis of *Bd* infection via histology is less sensitive than qPCR (Hyatt et al. 2007), results for the 102 histological samples we diagnosed may have suffered from a higher rate of false negatives than our qPCR samples. However, they form a relatively minor part of the data, and even if false negative rates for these samples are assumed to be high, the conclusion that prevalence of *Bd* infection in WT microhylids must be very low is not affected.

For 3 specific sites (Paluma, Site AU10 at Charmillin Creek on the Atherton Tableland, and Mt. Bellenden Ker), sample sizes from the swab surveys are sufficient in themselves to demonstrate that the prevalence of *Bd* in microhylids is very low, if it is present at all. The fungus has been present in stream-associated frogs in all 3 areas for a substantial period of time. At least 2 frog species (*Litoria nannotis* and *L. serrata*) have been found infected with *Bd* at Paluma since at least 1990 (Murray et al. 2010). Lips et al. (2006) showed that within a few months of the emergence of chytridiomycosis at a site in Panama, it had spread throughout the amphibian fauna, including species not closely associated with streams. By the early 1990s, numerous declines of frog populations had occurred on the Atherton Tablelands in the region of Site AU10 (Richards et al. 1993); these have subsequently been attributed to chytridiomycosis. Histological examination confirmed the presence of the fungus at multiple sites on the Atherton Tableland in the late 1990s and early 2000s (Murray et al. 2010). Frogs declined in the rainforest block containing Mt. Bellenden Ker in the early 1990s (Richards et al. 1993), and *Bd* has been present there since at least 1999 (Murray et al. 2010). The positive record for *Cophixalus ornatus* at Babinda Creek (Kriger & Hero 2006) is from a stream that drains this rainforest block. These results make it clear that the absence, or extremely low prevalence, of infections by *Bd* in the frogs at these widely separated sites must reflect the biology of Australian microhylid frogs.

One possible explanation for the absence or low prevalence of *Bd* infection could be the terrestrial habits of Australian microhylids; they might simply not come into contact with fungal zoospores. How-

ever, several species of exclusively terrestrial, direct-developing frogs do suffer from chytridiomycosis in the wild (Lips et al. 2006, Longo & Burrowes 2010). Lips et al. (2006) suggested that chytridiomycosis spreads by a combination of frog–frog and environment–frog transmission. These modes of transmission have been demonstrated in the laboratory (Davidson et al. 2003), and in field mesocosms (Parris & Cornelius 2004). Longo et al. (2010) demonstrated that 2 terrestrial direct-developing species exhibited clumping behaviour in retreat sites that could facilitate the transmission of *Bd*. *Bd* has been found on moist rocks, sticks, and leaves during an epidemic, supporting the hypothesis that it can be transmitted by contaminated environmental substrates (Lips et al. 2008, Richards-Zawacki 2010). Rowley & Alford (2007) showed that stream-associated frogs of susceptible species move long distances from water, which should disperse *Bd* zoospores onto substrates encountered by microhylids. All in all, Australian microhylids should not completely escape infection through lack of opportunity for transmission.

If there is more than very occasional transmission of *Bd* in nature, our results, in conjunction with the fact that the species most common in our data can become infected by *Bd* (Kriger & Hero 2006, Hauselberger 2011), suggest that Australian microhylids are likely to have high rates of clearance of infections. The only monitored populations have shown no signs whatever of declines (Hauselberger & Alford 2005), despite the fact that *Bd* has been established in their locality since the early 1990s. Declines should have occurred if transmission was common and infected individuals suffered rapid mortality. If transmission was common and infections were tolerated, we should have detected relatively high prevalences of infection, as are found in several species of WT hylids (Woodhams & Alford 2005, Puschendorf et al. 2011). Rates of clearance of infection can be determined by many factors, including environmental conditions, host behaviour, and pathogen and host biology. The prevalence of infection by *Bd* in WT hylid frogs undergoes seasonal changes, decreasing substantially in warmer months (Berger et al. 2004, Woodhams & Alford 2005, Kriger & Hero 2007). In the present study, field sampling occurred in the summer wet season from October to March. It is possible that because sampling was carried out in summer, prevalence of *Bd* infection was reduced, so that our results underestimate mean annual *Bd* prevalence. However, in montane regions, summer maximum temperatures may never attain levels lethal to *Bd*, and optimal chytrid growth temperatures may actually occur in summer. In the Australian WT, seasonal fluctuations tend to be less pronounced, and summer prevalences tend to be greater, at higher (>400 m) elevations (Woodhams & Alford 2005). In the present study, 563 of the 595 samples (95%) were collected at locations above 900 m. At an elevation lower than this, Puschendorf et al. (2011) found prevalences of 69% and greater during summer in susceptible species of stream-associated frogs. Disease prevalence in direct-developing frogs of Puerto Rico is actually higher during the warm-wet months (Longo et al. 2010).

Studies of the biology of *Bd in vitro* indicate that *Bd* is able to survive and grow between 4 and 28°C, but that the best conditions for growth occur between 17 and 25°C, with optimal growth at 23°C (Longcore et al. 1999, Piotrowski et al. 2004). The surface temperatures of frogs measured in the present study ranged from 10.8 to 24.3°C. All were within the envelope that supports *Bd* growth, and almost all were within the range in which the fungus grows at the highest rates. It is thus unlikely that the body temperatures of microhylids, even in the summer months, are sufficiently high to inhibit *Bd*.

Innate or acquired immune responses could provide microhylids with inherent protection from the effects of infection by *Bd* and high rates of clearance of infections. Innate immune defences may be important in reducing susceptibility to chytridiomycosis in terrestrial amphibian species that brood their eggs. These species may have particularly effective innate immune defences such as antifungal skin secretions or microbial symbionts as a means to reduce fungal infestations that would otherwise overwhelm egg clutches (Lauer et al. 2007). Peptide mixtures secreted by a range of amphibian species can inhibit *Bd* growth (Rollins-Smith et al. 2002, Woodhams et al. 2006). Antimicrobial skin flora are also likely to have a major role in determining the susceptibility of frogs to chytridiomycosis, and several brooding species of terrestrial salamanders possess diverse bacterial flora that inhibit the growth of microbes and fungi, including *Bd* (Harris et al. 2006, Lauer et al. 2007).

Amphibian declines caused by chytridiomycosis probably result from a complex web of factors including amphibian behaviour, environmental conditions, host immune function, and microbial symbionts. Further research into the biology of Australian microhylids is required to determine the causes of the very low prevalence of *Bd* infection in this family, and to determine whether those causes may suggest means of managing the pathogen's impact on more susceptible taxa.

Acknowledgements. This research was supported by funding from the Australian Rainforest Co-operative Research Centre and the Australian Government Department of Environment and Heritage (RFT 43-04; Primary investigator: R.A.A.), and was carried out under Scientific Purposes permits issued by the Queensland Parks and Wildlife Service (WISP02464008 and WITK 02464108), and a Permit to Collect issued by the Queensland Environmental Protection Agency (ATH 08/005) as approved by the James Cook University Animal Care and Ethics Committee (A931 and A960). K.F.H. was supported by an Australian Postgraduate Research Scholarship. Thanks to A. Hyatt, R. Campbell, S. Garland, and A. Phillott for analysis of qPCR samples. Thanks to T. Tantar, R. Hobbs, C. Steel, S. Bell, and many wonderful volunteers for assistance in the field.

LITERATURE CITED

Bell BD, Carver S, Mitchell NJ, Pledger S (2004) The recent decline of a New Zealand endemic: how and why did populations of Archey's frog *Leiopelma archeyi* crash over 1996-2001? Biol Conserv 120:189–199

Berger L, Speare R, Daszak P, Green DE and others (1998) Chytridiomycosis causes amphibian mortality associated with population declines in the rainforests of Australia and Central America. Proc Natl Acad Sci USA 95: 9031–9036

Berger L, Speare R, Kent A (2000) Diagnosis of chytridiomycosis in amphibians by histological examination. Zoos Print J 15:184–190 www.zoosprint.org

Berger L, Speare R, Hines HB, Marantelli G and others (2004) Effect of season and temperature on mortality in amphibians due to chytridiomycosis. Aust Vet J 82: 434–439

Berger L, Marantelli G, Skerratt LF, Speare R (2005) Virulence of the amphibian chytrid fungus *Batrachochytrium dendrobatidis* varies with the strain. Dis Aquat Org 68: 47–50

Boyle DG, Boyle DB, Olsen V, Morgan JAT, Hyatt AD (2004) Rapid quantitative detection of chytridiomycosis (*Batrachochytrium dendrobatidis*) in amphibian samples using real-time Taqman PCR assay. Dis Aquat Org 60:141–148

Brem FMR, Lips KR (2008) *Batrachochytrium dendrobatidis* infection patterns among Panamanian amphibian species, habitats and elevations during epizootic and enzootic stages. Dis Aquat Org 81:189–202

Brooke PN, Alford RA, Schwarzkopf L (2000) Environmental and social factors influence chorusing behaviour in a tropical frog: examining various temporal and spatial scales. Behav Ecol Sociobiol 49:79–87

Burrowes PA, Rafael LJ, Green DE (2004) Potential causes for amphibian declines in Puerto Rico. Herpetologica 60: 141–154

Bustamante HM, Livo LJ, Carey C (2010) Effects of temperature and hydric environment on survival of the Panamanian golden frog infected with a pathogenic chytrid fungus. Integr Zool 5:143–153

Cheng TL, Rovito SM, Wake DB, Vredenburg VT (2011) Coincident mass extirpation of neotropical amphibians with the emergence of the infectious fungal pathogen *Batrachochytrium dendrobatidis.* Proc Natl Acad Sci USA 108:9502–9507

Cogger HG (1996) Reptiles and amphibians of Australia. Reed Books, Chatswood

Daszak P, Berger L, Cunningham AA, Hyatt AD, Green DE, Speare R (1999) Emerging infectious diseases and amphibian population declines. Emerg Infect Dis 5: 735–748

Daszak P, Cunningham AA, Hyatt AD (2003) Infectious disease and amphibian population declines. Divers Distrib 9:141–150

Davidson EW, Parris M, Collins JP, Longcore JE, Pessier JP, Brunner J (2003) Pathogenicity and transmission of chytridiomycosis in tiger salamanders (*Ambystoma tigrinum*). Copeia 2003:601–607

Department of the Environment and Heritage (2006) Threat abatement plan: infection of amphibians with chytrid fungus resulting in chytridiomycosis. Department of Environment and Heritage, Canberra. Available at: www.environment.gov.au/biodiversity/threatened/publications/tap/chytrid.html

Felton A, Alford RA, Felton AM, Schwarzkopf L (2006) Multiple mate choice criteria and the importance of age for male mating success in the microhylid frog, *Cophixalus ornatus.* Behav Ecol Sociobiol 59: 786–795

Forester DC (1979) The adaptiveness of parental care in *Desmognathus ochrophaeus* (Uredola: Plethodontidae). Copeia 1979:332–341

Harris RN, James TY, Lauer A, Simon MA, Patel A (2006) Amphibian pathogen *Batrachochytrium dendrobatidis* is inhibited by the cutaneous bacteria of amphibian species. EcoHealth 3:53–56

Hauselberger KF (2001) The ecology and vocal behaviour of the Australian microhylid frog, *Austrochaperina robusta*, with comparisons to the sympatric species *Cophixalus ornatus*. Honours dissertation, James Cook University, Townsville

Hauselberger KF (2011) Ecology of microhylid frogs in the Australian Wet Tropics and implications for their vulnerability to chytridiomycosis. PhD dissertation, James Cook University, Townsville

Hauselberger KF, Alford RA (2005) Effects of season and weather on calling in the Australian microhylid frogs, *Austrochaperina robusta* and *Cophixalus ornatus.* Herpetologica 61:349–363

Hudson P, Rizzoli A, Grenfell B, Heesterbeek H, Dobson A (2001) The ecology of wildlife diseases. Oxford University Press, Oxford

Hyatt AD, Boyle DG, Olsen V, Boyle DB and others (2007) Diagnostic assays and sampling protocols for the detection of *Batrachochytrium dendrobatidis.* Dis Aquat Org 73:175–192

Kriger KM, Hero JM (2006) *Cophixalus ornatus* (ornate nursery frog), chytridiomycosis. Herpetol Rev 37:5

Kriger KM, Hero JM (2007) Large-scale seasonal variation in the prevalence and severity of chytridiomycosis. J Zool 271:352–359

Kriger KM, Hines HB, Hyatt AD, Boyle DG, Hero JM (2006) Techniques for detecting chytridiomycosis in wild frogs: comparing histology with real-time Taqman PCR. Dis Aquat Org 71:141–148

Lauer A, Simon MA, Banning JL, Andre E, Duncan K, Harris RN (2007) Common cutaneous bacteria from the eastern red-backed salmander can inhibit pathogenic fungi. Copeia 2007:630–640

Lips KR, Green DE, Papendick R (2003) Chytridiomycosis in wild frogs from southern Costa Rica. J Herpetol 37: 215–218

Lips KR, Brem F, Brenes R, Reeve JD and others (2006) Emerging infectious disease and the loss of biodiversity in a neotropical amphibian community. Proc Natl Acad Sci USA 103:3165–3170

Lips KR, Diffendorfer JE, Mendelson JR, Sears MW (2008) Riding the wave: reconciling the roles of disease and climate change in amphibian declines. PLoS Biol 6:441–454

Longcore JE, Pessier AP, Nichols DK (1999) *Batrachochytrium dendrobatidis* gen. et sp. nov., a chytrid pathogenic to amphibians. Mycologia 91:219–227

Longo AV, Burrowes PA (2010) Persistence with chytridiomycosis does not assure survival of direct-developing frogs. EcoHealth 7:185–195

Longo AV, Burrowes PA, Joglar RL (2010) Seasonality of *Batrachochytrium dendrobatidis* infection in direct-developing frogs suggests a mechanism for persistence. Dis Aquat Org 92:253–260

McDonald KR, Alford RA (1999) A review of the declining frogs in Northern Queensland. In: Campbell A (ed) Declines and disappearances of Australian frogs. Environment Australia, Canberra, p 14–22

McDonald KR, Mendez D, Muller R, Freeman AB, Speare R (2005) Decline in the prevalence of chytridiomycosis in frog populations in North Queensland, Australia. Pac Conserv Biol 11:114–120

Murphy PJ, St-Hilaire S, Corn PS (2011) Temperature, hydric environment, and prior pathogen exposure alter the experimental severity of chytridiomycosis in boreal toads. Dis Aquat Org 95:31–42

Murray K, Retallick R, McDonald KR, Mendez D and others (2010) The distribution and host range of the pandemic disease chytridiomycosis in Australia, spanning surveys from 1956–2007. Ecology 91:1557–1558

North S, Alford RA (2008) Infection intensity and sampling locality affect *Batrachochytrium dendrobatidis* distribution among body regions on green-eyed tree frogs *Litoria genimaculata.* Dis Aquat Org 81:177–188

Parris MJ, Cornelius TO (2004) Fungal pathogen causes competitive and developmental stress in larval amphibian communities. Ecology 85:3385–3395

Pessier AP, Nichols DK, Longcore JE, Fuller MS (1999) Cutaneous chytridiomycosis in poison dart frogs (*Dendrobates* spp.) and White's tree frogs (*Litoria caerulea*). J Vet Diagn Invest 11:194–199

Piotrowski JS, Annis SL, Longcore JE (2004) Physiology of *Batrachochytrium dendrobatidis*, a chytrid pathogen of amphibians. Mycologia 96:9–15

Pounds JA, Bustamante MR, Coloma LA, Consuegra JA and others (2006) Widespread amphibian extinctions from epidemic disease driven by global warming. Nature 439: 161–167

Puschendorf R, Hoskin CJ, Cashins SD, McDonald KR and others (2011) Environmental refuge from disease-driven amphibian extinction. Conserv Biol 25:956–964

Retallick RW, McCallum H, Speare R (2004) Endemic infection of the amphibian chytrid fungus in a frog community post-decline. PLoS Biol 2:1965–1971

Richards DG, McDonald KR, Alford RA (1993) Declines in populations of Australia's endemic tropical rainforest frogs. Pac Conserv Biol 1:66–77

Richards-Zawacki CL (2010) Thermoregulatory behaviour affects prevalence of chytrid fungal infection in a wild population of Panamanian golden frogs. Proc R Soc Lond B Biol Sci 277:519–528

Rollins-Smith LA, Doersam JK, Longcore JE, Taylor SK, Shamblin JC, Carey C, Zasloff MA (2002) Antimicrobial peptide defenses against pathogens associated with global amphibian declines. Dev Comp Immunol 26:63–72

Rowley JJL, Alford RA (2007) Behaviour of Australian rainforest stream frogs may affect the transmission of chytridiomycosis. Dis Aquat Org 77:1–9

Sanchez D, Chacon-Ortiz A, Leon F, Han B, Lampo M (2008) Widespread occurrence of an emerging pathogen in amphibian communities of the Venezuelan Andes. Biol Conserv 141:2898–2905

Schloegel LM, Hero JM, Berger L, Speare R, McDonald KR, Daszak P (2006) The decline of the sharp-snouted day frog (*Taudactylus acutirostris*): the first documented case of extinction by infection in a free-ranging wildlife species? EcoHealth 3:35–40

Simon MP (1983) The ecology of parental care in a terrestrial breeding frog from New Guinea. Behav Ecol Sociobiol 14:61–67

Skerratt LF, Berger L, Speare R, Cashins S and others (2007) Spread of chytridiomycosis has caused the rapid global decline and extinction of frogs. EcoHealth 4:125–134

Soto-Azat C, Clarke BT, Fisher MC, Walker SF, Cunningham AA (2009) Non-invasive sampling methods for the detection of *Batrachochytrium dendrobatidis* in archived amphibians. Dis Aquat Org 84:163–166

Speare R, Berger L, Skerratt LF, Alford RA and others (2004) Hygiene protocol for handling amphibians in field studies. Amphibian Diseases Group, James Cook University, Townsville

Stockwell MP, Clulow J, Mahony M (2010) Host species determines whether infection load increases beyond disease-causing thresholds following exposure to the amphibian chytrid fungus. Anim Conserv 13:62–71

Van Riper C III, Van Riper SG, Goff ML, Laird M (1986) The epizootiology and ecological significance of malaria in Hawaiian land birds. Ecol Monogr 56:327–344

Van Sluys M, Kriger KM, Phillott AD, Campbell R, Skerratt LF, Hero JM (2008) Storage of samples at high temperatures reduces the amount of amphibian chytrid fungus *Batrachochytrium dendrobatidis* DNA detectable by PCR assay. Dis Aquat Org 81:93–97

Wakelin D (1978) Genetic control of susceptibility and resistance to parasitic infection. Adv Parasitol 16:219–308

Warner RE (1968) The role of introduced diseases in the extinction of the endemic Hawaiian avifauna. Condor 70: 101–120

Weldon C, du Preez LH, Hyatt AD, Muller R, Speare R (2004) Origin of the amphibian chytrid fungus. Emerg Infect Dis 10:2100–2105

Williams YM, Williams SE, Alford RA, Waycott M, Johnson CN (2006) Niche breadth and geographical range: ecological compensation for geographical rarity in rainforest frogs. Biol Lett 2:532–535

Woodhams DC, Alford RA (2005) Ecology of chytridiomycosis in rainforest stream frog assemblages of tropical Queensland. Conserv Biol 19:1449–1459

Woodhams DC, Alford RA, Marantelli G (2003) Emerging disease of amphibians cured by elevated body temperature. Dis Aquat Org 55:65–67

Woodhams DC, Rollins-Smith LA, Carey C, Reinhart LK, Tyler MJ, Alford RA (2006) Population trends associated with skin peptide defenses against chytridiomycosis in Australian frogs. Oecologia 146:531–540

Editorial responsibility: John Austin, Oldendorf/Luhe, Germany

Submitted: November 18, 2011; Accepted: June 15, 2012
Proofs received from author(s): August 23, 2012