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Retreat sites of rain forest stream frogs are not a reservoir for *Batrachochytrium dendrobatidis* in northern Queensland, Australia

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ABSTRACT: Chytridiomycosis is a potentially fatal disease of amphibians caused by *Batrachochytrium dendrobatidis*, and is implicated in declines and extinctions of amphibian populations and species around the world. To cause local host extinction, a disease organism must persist at low host densities. One mechanism that could facilitate this is the ability to persist in the environment. In the laboratory, *B. dendrobatidis* spreads by both frog-to-frog and environment-to-frog transmission, and can persist on a number of biotic substrates. In the field, *B. dendrobatidis* has been detected on environmental samples taken during an epidemic, but it is not known if it persists in the environment when endemic. Retreat sites of 2 species of Australian rain forest stream frogs *Litoria lesueuri* and *L. nannotis* were sampled 0 to 3 d after occupation during the wet and dry seasons in northern Queensland, Australia, where chytridiomycosis has been endemic for at least 10 yr. The intensity and prevalence of infection in frogs during sampling were comparatively low compared with epidemics. Diagnostic quantitative polymerase chain reaction did not detect *B. dendrobatidis* in any retreat site samples. It thus appears that retreat sites are not a major environmental source of infection when *B. dendrobatidis* occurs at low prevalence and intensity on frogs. This suggests that control efforts may not need to eliminate the organism from the environment, at least when prevalence and intensity of infection are low in frogs. Simply treating hosts may be effective at controlling the disease in the wild.

KEY WORDS: *Batrachochytrium dendrobatidis* · Amphibian chytrid fungus · Chytridiomycosis · Environmental reservoir · Disease transmission · Frogs

INTRODUCTION

Amphibian species around the world are declining at an alarming rate, many of them to extinction (Blaustein & Wake 1990, Alford & Richards 1999, Kiesecker et al. 2001, Stuart et al. 2004). Currently, approximately 43% of all known amphibian species are considered to be declining (Stuart et al. 2004). Many of these declines are thought to be due to the amphibian disease chytridiomycosis (Berger et al. 1998, Lips et al. 2006), which can be fatal to many species and is implicated in mass mortalities, population declines and extinctions in Australia, New Zealand, Central and North America, Europe and Africa (Berger et al. 1998, Lips 1999, Bosch et al. 2001, Bradley et al. 2002, Muths et al. 2003, Bell et al. 2004, Weldon & Du Preez 2004, Lips et al. 2005, 2006).

Chytridiomycosis is caused by the pathogen *Batrachochytrium dendrobatidis*, which belongs to the order Chytridiales (Longcore et al. 1999). Members of this order, commonly referred to as chytrids, are ubiquitous fungi found in aquatic habitats and moist soils (Sparrow 1960). They occur as saprobes or parasites on a wide range of substrates including algae, other aquatic fungi, aquatic and terrestrial plants, spores, microscopic animals and their eggs, and chitinous insect exoskeletons (Sparrow 1960), and subsist by degrading cellulose, chitin and keratin (Powell 1993). A number of chytrids are parasitic, infecting plants, algae, protists, invertebrates and vertebrates (Powell 1993).

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B. dendrobatidis is the only chytrid known to cause disease in vertebrate hosts (Berger et al. 1998), presumably breaking down keratin that occurs in the epidermis of adult amphibians and the mouthparts of larval anurans. Chytridiomycosis can cause rapid mortality (Nichols et al. 2001), with infected frogs of susceptible species dying within 3 wk of infection in the laboratory (Berger et al. 1998, 2004). In the laboratory, the disease is highly contagious (Nichols et al. 2001), spreading within and among individuals via motile, waterborne zoospores (Longcore et al. 1999).

One of the unusual aspects of chytridiomycosis is that it drives many host species to local extinction during outbreaks (Berger et al. 1998, Lips et al. 2006). For a pathogen to cause local host extinction it must be capable of persisting and infecting new hosts even at very low host population densities (Anderson & May 1986, Dobson & May 1986). One potential mechanism for this is the persistence and growth of *Batrachochytrium dendrobatidis* in tadpoles and adults that do not die from infection (Berger et al. 1998, 1999, 2004, Daszak et al. 1999). The broad host range of *B. dendrobatidis* also provides a reservoir of infection, enabling the species most susceptible to chytridiomycosis to be driven to extinction (Berger et al. 2004). An alternative source of infection that could facilitate extinction is the persistence of free-living stages (Daszak & Cunningham 1999, Godfray et al. 1999).

A number of aquatic pathogens are able to persist as viable organisms in the environment by forming biofilms on both abiotic and biotic surfaces (Carli et al. 1993, Hood & Winter 1997, Signoretto et al. 2005). Zoospores of many chytrids can persist in films of water on plants and in soil, and in ponds and rivers (Carlile & Watkinson 1994), and have been detected on mossy rocks (Dewel et al. 1985) and canopy leaves (Longcore 2005). In the laboratory, *Batrachochytrium dendrobatidis* can be cultured on tryptone agar without keratin or keratin derivatives (Longcore et al. 1999, Pessier et al. 1999), will persist in sterilised water for several weeks and will grow for at least one generation on cleaned epidermal keratin or dead amphibians (Longcore et al. 1999). In addition, zoosporangia can attach to and grow on dead algae and insect exoskeletons (Johnson & Speare 2003) and survive for at least 3 mo in sterile sand or bird feathers (Johnson & Speare 2005). Another source of infection that could facilitate extinction is the ability of *B. dendrobatidis* to infect alternative hosts. All these factors indicate that *B. dendrobatidis* may be able to do some or all of the following: persist for some time in the environment, grow saprophytically, or infect alternate hosts (Longcore et al. 1999).

Various modes of transmission have been demonstrated in the laboratory and in mesocosms. Direct transmission is known to occur via contact from frog-to-frog (G. Marantelli pers. comm.), while indirect transmission can occur between frogs via substrates in mesocosms (G. Marantelli pers. comm.) and from frog to water to frog or tadpole (Berger et al. 1998, Parris & Cornelius 2004, Rachowicz & Vredenburg 2004).

During a recent mass mortality event in Panama, Lips et al. (2006) detected *Batrachochytrium dendrobatidis* via PCR (polymerase chain reaction) on 6 of 7 substrate samples associated with dead frogs and on 1 of 9 stream boulders. However, it is not known if *B. dendrobatidis* is present on substrates only during epidemics, nor is it known whether *B. dendrobatidis* persists on these substrates or if transmission to amphibians occurs.

If *Batrachochytrium dendrobatidis* can persist or grow in the environment, this will be an important source of infection, particularly if environmental persistence occurs in sites where frogs spend large proportions of their time, such as retreat sites. Particularly during daylight hours, nocturnal frogs usually remain motionless in species-specific retreat sites. Most species of Australian rain forest stream frog occupy these sites from 12 h to 5 d at a time, and can return to them repeatedly (J. J. L. Rowley & R. A. Alford unpubl. data). Retreat sites, therefore, might provide opportunities for reinfection of individuals and transmission between individuals. This could be particularly important because the highest intensities of infection with *B. dendrobatidis* occur on the ventral surfaces of frogs (Berger et al. 2005), which are most often in contact with substrates. We examined the possibility that reinfection and transmission might occur via contaminated substrates by using diagnostic PCR to determine if *B. dendrobatidis* persisted in the diurnal retreat sites of 2 species of rain forest stream frog known to be infected with *B. dendrobatidis* in the field.

MATERIALS AND METHODS

The diurnal retreat sites used by most species of frogs are unknown. We located the diurnal retreat sites used by 2 species of frogs (*Litoria lesueuri* and *L. nannotis*) by tracking individuals at 3 relatively undisturbed rain forest streams in northern Queensland, Australia; Frenchman Creek, in Wooroonooran National Park (17° 20′ S, 145° 55′ E, 20 to 100 m above sea level [asl]), and Python Creek (17° 46′ S, 145° 35′ E, 200 m asl) and an unnamed creek ('Lower Tully Creek', 17° 48′ S, 145° 41′ E, 70 m asl) in Tully Falls Forest Reserve. Surveys were conducted in both the wet and the dry seasons at each site; each survey was approximately 16 d in duration. Mean air temperature in the wet season was 25°C (range = 21 to 32°C) and in the dry season was 18°C (range = 13 to 25°C).

During the study, we tracked large to medium-sized hylid frogs of the 2 species *Litoria lesueuri* and *Litoria nannotis* and swabbed their diurnal retreat sites. Recently, the taxonomy of the *L. lesueuri* group has been revised (Donnellan & Mahony 2004). Two species, *L. jungguy* and *L. wilcoxii*, occur in sympatry in the region, may hybridise, and are indistinguishable on the basis of morphology (Donnellan & Mahony 2004). We therefore continue to refer to them as *L. lesueuri*. We tracked *L. nannotis* at Python Creek from 23 July to 6 August 2004 and 25 March to 8 April 2004, and *L. lesueuri* at Frenchman Creek from 15 to 29 March and 3 to 17 August 2005 and at Lower Tully Creek from 22 February to 9 March and 25 August to 9 September 2005. Frogs were tracked using either radio telemetry or harmonic radar direction finding (Langkilde & Alford 2002). Only frogs weighing >11 g were tracked via radio telemetry. Radio transmitters (Models BD-2N and BD-2NT; Holohil Systems; weighing approximately 0.67 g, including harness, and with a battery life of approximately 3 wk) were attached to a harness made of silicone tubing, designed to minimise restrictions on movement and avoid causing discomfort to the frog. Frogs that were too small to be radio tracked, as well as a number of larger individuals, were tracked using harmonic radar tracking. This required attachment of a small diode to the same specially designed harness (weighing approximately 0.27 g, including harness). Frogs were fitted with tracking devices *in situ* and released at their point of capture in <5 min. Tracking devices and harnesses did not weigh >6% of the frogs' total body weights, which is just over half the recommended maximum relative weight for an attached tag (10% of the body weight; Richards et al. 1994). The weights of frogs tracked did not change over the study period (Wilcoxon's signed ranks test; $Z = -1.361$, $p = 0.173$, $n = 70$), and frogs with tracking devices attached appeared to use retreat sites similarly to individuals without tracking devices; tracked frogs were commonly observed in close association with, and sharing retreat sites with, frogs without tracking devices.

Frogs fitted with radio transmitters were tracked using a 3-element folding Yagi antennae (A.F. Antronics) and Habit Research HR2500 Osprey VHF receivers. Frogs fitted with diodes were tracked using a portable RECCO R5 transmitter-receiver unit (Recco Rescue Systems).

After we located a frog in a diurnal retreat site, we flagged the site, photographed its position, and constructed a detailed diagram allowing us to accurately relocate the site. We returned to retreat sites approximately 24, 48 and 72 h after initial location, and, if the frog had left, we swabbed its exact previous location 5 times with a sterile cotton swab (Medical Wire & Equipment Co.).

Diurnal retreat sites for *Litoria lesueuri* were typically on the ground, on leaf litter, gravel, soil, or clay, and it was possible to sample almost every site. In contrast, diurnal retreat sites for *L. nannotis* were typically inaccessible, being under large boulders, in rock fissures, or in caves. While it was possible to swab individual retreat sites of *L. lesueuri* over time, it was not possible to swab most of the *L. nannotis* retreat sites found during 2004. We therefore focused on 2 accessible sites under waterfalls at Python Creek, where we always found aggregations of *L. nannotis* during the day, and swabbed these sites during 2005, at intervals of approximately 4 d. Although we attempted to relocate each *L. lesueuri* retreat site every 24 h, there were a number of instances when we were unable to relocate the retreat site of a frog.

Individual *Litoria lesueuri* were swabbed pre- and post-tracking by swabbing their ventral surface, hands and feet with a sterile cotton swab. These samples and samples taken from retreat sites were evaluated for the presence of *Batrachochytrium dendrobatidis* using Taqman diagnostic quantitative PCR (Boyle et al. 2004). DNA was extracted with PrepMan Ultra and amplified using the primers ITSI-3 Chytr and 5.8S Chytr (Boyle et al. 2004). Each sample was tested in triplicate, and a sample was only recorded as positive if all 3 replicates indicated the presence of *B. dendrobatidis*. If only 1 or 2 replicates were positive for the presence of *B. dendrobatidis*, the sample was regarded as a 'suspicious positive' and retested. As a large number of samples (>400) were collected from *L. lesueuri* retreat sites, we only evaluated samples collected from sites used by frogs that tested positive or suspicious positive for *B. dendrobatidis.*

RESULTS

Infection prevalence was low for *Litoria lesueuri* during the survey periods. At Frenchman Creek, infection prevalence was only 4.5% (n = 22) during the wet season surveys and 7.7% (n = 26) during the dry season surveys. Similarly, at Lower Tully Creek, infection prevalence was 6.3% (n = 16) during surveys in the wet season surveys and 17.4% (n = 23) during the dry season surveys. In frogs that tested positive or suspicious positive for *Batrachochytrium dendrobatidis*, infection intensity was also low (Table 1).

Of the 81 retreat site swabs from 36 *Litoria lesueuri* retreat sites, *Batrachochytrium dendrobatidis* was detected using quantitative PCR from only 1 sample (1.2% of samples tested). Of the 41 swabs taken of *L. nannotis* retreat sites, *B. dendrobatidis* was detected in 3 samples (7.3% of the retreat sites tested). Because all detected levels of *B. dendrobatidis* were very low,

Table 1. Presence of *Batrachochytrium dendrobatidis* at retreat sites 0 to 3 d after use by *Litoria lesueuri* that tested positive or suspicious positive for *B. dendrobatidis*. –: no data

Season	Site	Frog ID no.	Intensity of *B. dendrobatidis* infection (estimated no. of zoospore genomes swab^{-1})		Days since use	Number of retreat sites testing	
			Pre-tracking	Post-tracking		Negative	Positive
Wet	Frenchman Creek	1	<1[a]	<1[a]	1	1	0
					3	1	0
	Lower Tully Creek	2	6	4[a]	1	4	0
					2	3	0
Dry	Frenchman Creek	3	57	–	1	3	0
					2	2	0
					3	1	0
					1[b]	1	0
		4	2327	–	1	2	0
					2	2	0
	Frenchman Creek	5	<1[a]	–	1	5	0
	Lower Tully Creek				2	4	0
					3	3	0
		6	<1	0	0	1	0
					1	4	0
					2	4	0
					3	2	0[c]
					1[b]	1	0
		7	0	498	0	0	0
					1	1	0
					2	2	0
					3	2	0
		8	1[a]	0	0[b]	1	0
					1	5	0
					2	5	0
					3	2	0
		9	8	41	1	4	0
					2	4	0
					3	4	0
		10	121	–	1	1	0
					2	3	0
					3	3	0
Total						81	0

[a]Suspicious positive
[b]Frog present at retreat site for over 48 h
[c]Initially 1 sample tested positive for *B. dendrobatidis* in 1 of 3 wells, estimated <1 zoospore genome equivalent present; retested negative in all 3 wells

and no swabs tested positive on all 3 replicate tests, we regarded these only as suspicious positives and re-tested them. On retesting, all samples were negative for *B. dendrobatidis* in all 3 wells (Tables 1 & 2).

DISCUSSION

The retreat sites we tested did not harbour *Batrachochytrium dendrobatidis* within 0 to 3 d after use, as determined by swabbing and diagnostic PCR. It appears that retreat sites are not a reservoir of infection when *B. dendrobatidis* occurs at low prevalence and intensity on frogs. It is also likely that they are not a major mechanism of transmission within populations where *B. dendrobatidis* occurs at low prevalence and intensity, unless transmission occurs shortly after (<12 h) *B. dendrobatidis* zoospores are shed at a retreat site by an infected frog.

Both the prevalence and intensity of infection were low for *Litoria lesueuri* when retreat sites were sampled. The prevalence and intensity of infection for *L. nannotis* at the time of the retreat site survey are unknown; however, in a creek adjacent to Python Creek at similar times of the year, they were 50% and 35 zoospore genome equivalents per swab, respectively, in the dry season, and 17% and 5 zoospore genome equivalents per swab in the wet season (K. R. McDonald & L. F. Skerratt un-

Table 2. Presence of *Batrachochytrium dendrobatidis* at retreat sites after use by *Litoria nannotis* at Python Creek, Tully Gorge

Season	Location	Date 2005	Number of retreat sites testing	
			Negative	Positive
Wet	1	24 Feb	6	0[a]
		28 Feb	4	0[a]
		4 Mar	4	0[b]
	2	24 Feb	6	0
Dry	1	31 Aug	7	0
		4 Sep	8	0
		8 Sep	6	0
Total			41	0

[a]Initially tested positive for *B. dendrobatidis* in 1 of 3 wells, estimated <1 zoospore genome equivalent present; retested negative in all 3 wells
[b]Initially tested positive for *B. dendrobatidis* in 1 of 3 wells, estimated 4 zoospore genome equivalents present; retested negative in all 3 wells

publ. data). We suggest that retreat sites may become an important source of infection and reservoir when prevalence and intensity are higher, such as in recent outbreaks in Panama (Lips et al. 2006).

The survival of *Batrachochytrium dendrobatidis* in the environment is likely to vary due to differences in various abiotic and biotic factors. Laboratory studies have demonstrated that *B. dendrobatidis* growth and survival depend on substrate pH (Piotrowski et al. 2004, Johnson & Speare 2005) and the presence and composition of bacteria and oomycetes (Longcore et al. 1999, Harris et al. 2006). It is therefore possible that conditions were not optimal at our study sites and that variation in such factors may cause differential survival of *B. dendrobatidis* in the environment between sites. It is also possible that we failed to detect *B. dendrobatidis* DNA due to inhibition of the PCR, although we found no widespread evidence of inhibition.

The few low initial positive tests for retreat sites that turned out negative upon retesting might be explained by contamination of samples during loading of the PCR machine. This could be due to aerosolisation of DNA created during pipetting. Automation of this procedure and reduction in the number of standards in each PCR run might reduce contamination, as might automation of extraction procedures and conducting extractions on one sample at a time. We also include a positive control swab in each batch of 24 extractions immediately prior to the negative control swab during extractions and loading of samples into the PCR machine. It is possible that this control may contribute to contamination; however, it is important to maintain this control as it increases the sensitivity of the negative control to test for contamination during extractions and loading. To maximise our chances of detecting contamination of reagents at any step in the PCR, we also included a negative control that contains only the reagents in each PCR run. If either of the negative controls is positive, then all the samples are retested. Regardless of the cause, these findings demonstrate that low-level contamination of PCR tests can occur and that it is important to test samples in triplicate to ensure high specificity for the PCR test. Tests with only a proportion of replicates positive, especially when the concentration of detected DNA is low, should be regarded as 'suspicious positives' and should be retested if one wishes to confirm that they are positive.

Further work is required to determine whether retreat sites are important in the host–pathogen relationships between *Batrachochytrium dendrobatidis* and amphibians, particularly in regions and in species where the prevalence and intensity of *B. dendrobatidis* infection is high. Although *B. dendrobatidis* was not detected at frog retreat sites in the current study, it is still possible that the presence of an environmental reservoir is one factor causing the extremely high transmission rates seen in chytridiomycosis outbreaks in some systems (Berger et al. 1998, Lips et al. 2006). Our results, however, suggest that the environment may not provide a continual source of infection for amphibian species when the prevalence and intensity of infection with *B. dendrobatidis* are low. It may be possible to eliminate or greatly reduce the intensity of *B. dendrobatidis* in these situations by treating amphibians, without needing to decontaminate the environment, thereby enabling recolonisation or the successful release of captive-bred animals.

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