

# The chytrid fungus *Batrachochytrium dendrobatidis* is non-randomly distributed across amphibian breeding habitats

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## ABSTRACT

The chytrid fungus *Batrachochytrium dendrobatidis* has been implicated as the causative agent of mass mortalities, population declines, and the extinctions of stream-breeding amphibian species worldwide. While the factors that limit the distribution and abundance of *B. dendrobatidis* across large geographical regions are fairly well understood, little is known about the distribution of the fungus within localized areas such as individual catchments. The accurate identification of amphibian populations likely to be exposed to the fungus is urgently required for effective disease management. We conducted disease surveys of frogs representing five ecological guilds in south-east Queensland, Australia, and hypothesized that if *B. dendrobatidis* were responsible for the disappearance of stream-breeding amphibian populations, infection prevalence and intensity would be greatest in frogs breeding in permanent, flowing water. Overall, 30.3% of the 519 frogs we sampled were infected with *B. dendrobatidis*. However, infections were not evenly distributed across the ecological guilds, being almost completely restricted to frogs breeding at permanent waterbodies. Of these, stream breeders were significantly more likely to be infected than were pond breeders, though the intensity of frogs' infections did not differ significantly between the two guilds. *Batrachochytrium dendrobatidis* was detected on only one of the 117 frogs that were found at ephemeral ponds, ephemeral streams, or terrestrial sites. These findings provide strong support for the hypothesis that *B. dendrobatidis* was responsible for many of the unexplained disappearances of stream-breeding amphibian populations in recent decades, and will enable wildlife managers to more accurately focus conservation efforts on those species at highest risk of disease-related decline.

## Keywords

Amphibian declines, *Batrachochytrium dendrobatidis*, breeding habitat, chytrid, chytridiomycosis, population declines, wildlife disease.

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## INTRODUCTION

Amphibians are among the planet's most threatened taxa, with nearly one-third of the world's 6140 species threatened with extinction (Stuart *et al.*, 2004). Population declines in recent decades have been particularly severe, and the species extinction rate over the period 1980–2004 is thought to be up to 97 times higher than the background rate for the five centuries prior (Stuart *et al.*, 2004). Amphibians are considered to be accurate indicators of environmental health (Blaustein & Wake, 1995; Welsh & Droege, 2001), and their disappearance can lead to cascading effects that reverberate through the ecosystem (Ranvestel *et al.*, 2004; Whiles *et al.*, 2006). Amphibians also produce a wide array

of skin secretions, many of which have significant potential to improve human health through their use as pharmaceuticals (Traynor, 1998; VanCompernelle *et al.*, 2005). Furthermore, amphibians provide a source of enjoyment and cultural fascination to humans (Gibbons, 2003) and it would be morally irresponsible to allow them to continue on their current extinction trajectory (Mendelson *et al.*, 2006). For these reasons, it is imperative that the causative agents of amphibian declines and extinctions be identified and mitigated as rapidly as possible.

Amphibians exhibit a diverse array of breeding strategies (Duellman & Trueb, 1986; McDiarmid & Altig, 1999). Some species lay eggs in permanent ponds or streams and have aquatic larvae that may take years to metamorphose (Bury & Adams,

1999). Other species deposit their eggs in the water-filled leaf axils of plants, or ephemeral pools or streams that appear only after heavy rains. As these ephemeral habitats may dry soon after a breeding event, the aquatic larvae often metamorphose within a matter of days or weeks (Anstis, 2002). Still other species (terrestrial breeders) bypass the aquatic tadpole stage altogether, depositing their eggs in moist leaf-litter, where the young metamorphose within the eggs and emerge as tiny froglets (Ehmann & Swan, 1985).

With such different life histories, sympatric species can be exposed to very different threats. For instance, while prolonged droughts may result in failed recruitment for terrestrial breeders or species breeding in ephemeral waterbodies (Lips *et al.*, 2003a), species that spend a large proportion of their life in or near permanent water may be severely affected by waterborne pathogens, introduced fishes or pollutants (Laurance *et al.*, 1996; Gillespie, 2001; Rowe *et al.*, 2001). It is not surprising then that amphibian declines have not been randomly distributed across ecological guilds. Whereas only 24% of the world's 'rapidly declining' amphibian species prefer still waterbodies, 63% are associated with flowing water (Stuart *et al.*, 2004). In Australia, 58% of the amphibian species breeding in rainforest streams are threatened, vs. only 15% of the terrestrial species, and 9% of the pond-breeding species (Hero & Morrison, 2004), and an association with lotic streams is a significant predictor of declining population status (Williams & Hero, 1998). Association with aquatic habitats is a significant predictor of declining population status in Central American amphibian communities as well, though both pond- and stream-breeding species are more likely to decline than terrestrial breeders or species that breed in bromeliads (Lips, 1998; Lips *et al.*, 2003a). In Ecuador, Bustamante *et al.* (2005) re-surveyed seven sites with well-documented historical data, and found that while the species richness of direct-developing terrestrial species remained constant (28 species) across survey periods, the number of species with aquatic larvae declined by 50%, from 34 to only 17. These findings strongly suggest that one of the primary agents responsible for widespread amphibian declines in recent decades is waterborne, and more specifically, tends to be most prevalent in flowing water.

*Batrachochytrium dendrobatidis* is a parasitic chytrid fungus that infects the keratinized epidermis of amphibians and causes the potentially lethal skin disease chytridiomycosis (Berger *et al.*, 1998). The fungus has flagellated zoospores (Longcore *et al.*, 1999), cannot survive desiccation (Johnson *et al.*, 2003), and has been recovered from dead and dying amphibians at ponds and streams worldwide (Bosch *et al.*, 2001; Waldman, 2001; Muths *et al.*, 2003; Weldon *et al.*, 2004; Lips *et al.*, 2006; Schloegel *et al.*, 2006). As *B. dendrobatidis* is often found at sites concurrent with population decline, and as the physiology of *B. dendrobatidis* makes the fungus perfectly suited to aquatic habitats, chytridiomycosis has become the leading explanation put forth for the global decline of stream-breeding amphibian populations (Daszak *et al.*, 1999; La Marca *et al.*, 2005).

If chytridiomycosis were the primary cause of population declines in stream-breeding amphibians, we would expect that the prevalence and/or intensity of *B. dendrobatidis* infections in

this ecological guild would be higher than that for other guilds. Conversely, since there have been few reports of either chytridiomycosis or population decline in terrestrial-breeding amphibians, we would expect that the prevalence and/or intensity of chytrid infections in terrestrial breeders would be low. There exists, however, little empirical evidence to either support or refute these hypotheses, and there is no published work that explicitly examines this issue. To ameliorate this deficit in the literature, we conducted disease surveys in frog populations of the Numinbah Valley in south-east Queensland, Australia. Specifically, we aimed to determine to what extent *B. dendrobatidis* infection prevalence and intensity differ among the five ecological guilds represented in the valley: permanent-stream breeders, permanent-pond breeders, ephemeral-stream breeders, ephemeral-pond breeders, and terrestrial breeders.

## METHODS

### Field methods

The degree to which *B. dendrobatidis* infection levels vary across catchments is unknown, and sampling frogs from different catchments could confound results. As such, we restricted our sampling to a 22-km stretch of the Numinbah Valley, in the Nerang River catchment of south-east Queensland, Australia. All sampling took place within 1 km of the Nerang River or major tributaries. The proximity of all sites to each other ensured that any significant variation in chytrid levels between frogs of different ecological guilds would likely be due to the hydric requirements of the fungus and the life-history characteristics of the frogs, rather than to large-scale climatic differences between sites, different *B. dendrobatidis* introduction histories, or other unidentifiable site differences.

To control for any potentially confounding effects of altitude, we conducted all sampling in the lowlands, between 30 and 305 m a.s.l. To minimize any effect of seasonality, we sampled all frogs in the late winter and spring (8 September to 7 December), the period when chytrid levels in the valley are at their highest (Kriger & Hero, 2007). While sampling took place over three separate field seasons (2004–06), past work of ours (Kriger & Hero, 2007; K.M.K., unpub. data) has shown that there was no significant annual variation in chytrid levels in Numinbah Valley over the time period encompassed by our study. We therefore have no reason to believe that our results were biased by having sampled frogs in multiple years.

As the amount of time spent at breeding sites differs between male and female frogs, so may their chances of being exposed to the waterborne zoospores of *B. dendrobatidis*, and thus their chances of acquiring chytridiomycosis (Kagarize Sherman & Morton, 1993; Muths *et al.*, 2003). Furthermore, frogs of different life stages may vary in their susceptibility to chytrid infections (Lamirande & Nichols, 2002). We therefore restricted our sampling to adult males, the group of frogs most often encountered at sites. Females, juveniles, and metamorphs were encountered infrequently enough that had we sampled them we would not have been able to achieve statistically meaningful sample sizes. We

refrained from sampling tadpoles because a non-lethal sampling method did not exist at the time our study was conducted.

Frogs were located using visual and auditory techniques. At all sites, the first frogs that were encountered were captured, and collection ceased when either no more frogs could be found, or desired sample size ( $n = 30$ ) was achieved. Stream transects were conducted by walking alongside the edge of the stream, pond transects were conducted by circumambulating the pond's edge, and terrestrial sites were surveyed by randomly meandering through the forest until a calling frog's location could be determined via triangulation. The Nokia 5100 mobile telephone's 'mosquito' ringtone was used to elicit calling response in *Assa darlingtoni*. Frog surveys began just after sunset and generally ended by midnight.

Frogs were captured in clean, unused 20 × 25 cm plastic bags. We sampled each frog for *B. dendrobatidis* infection by firmly running a sterile cotton swab (MW 100–100, Medical Wire & Equipment, Corsham, Wiltshire, UK; Kriger *et al.*, 2006a) 10 times over each of the following locations: (1) the frog's dorsal surface, (2) the frog's sides, from groin to armpit, (3) the ventral surface, and (4) the undersides of the thighs. Additionally, five outward strokes of the swab were employed on the undersides of each frog's feet, for a total of 70 strokes. Swabs were then replaced in their original container (a plastic tube), and frozen at  $-20^{\circ}\text{C}$  upon return from the field. All frogs were handled with unused non-powdered latex gloves so as to prevent disease transmission between animals. All animals were released immediately after being sampled.

Frogs can move great distances away from their breeding site, and certain species can breed in multiple habitat types. As it is impossible to know where a frog was prior to its capture, we classified the breeding habitat of all frogs as the habitat type at which they were captured: permanent stream, permanent pond, ephemeral stream, ephemeral pond, or terrestrial. *Assa darlingtoni* is the only frog species in the Numinbah Valley that has direct-developing, non-aquatic larvae, and was thus the only frog classified in the last category.

### Laboratory analysis

Swabs were analysed for the presence of *B. dendrobatidis* using established quantitative (real-time) polymerase chain reaction (PCR) techniques (Boyle *et al.*, 2004), and employing the changes described by Kriger *et al.* (2006b). Thus, all samples that tested positive in the initial singlicate qPCR assay were re-analysed using a triplicate assay and a full set of *B. dendrobatidis* standards, in order to confirm the initial result and accurately quantify the number of *B. dendrobatidis* zoospores present.

### Data analysis

We assigned a positive infection status to any frog on whose swab at least one *B. dendrobatidis* zoospore equivalent was detected (Kriger *et al.*, 2007). Infection prevalence for frogs of each ecological guild was calculated by dividing the number of frogs positive for *B. dendrobatidis* by the total number of frogs

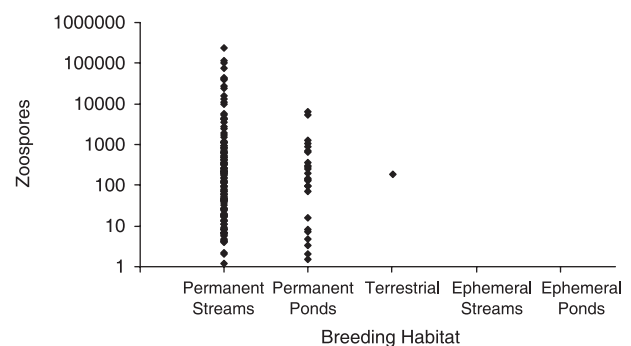
sampled. Chi-squared tests were used to test for significant differences in prevalence between the five groups. Quantification of chytrid zoospores on infected frogs is given as the mean value of *B. dendrobatidis* zoospore equivalents detected in the three replicates of the triplicate PCR analysis. We use this number as an index of the intensity of a frog's infection (parasite load). As the number of zoospores detected on infected frogs varied over five orders of magnitude, data were log transformed prior to performing statistical analyses. An independent *t*-test was used to test for significant differences in infection intensity between permanent-stream and permanent-pond breeding frogs, the only ecological guilds with sufficient numbers of infected frogs to allow statistical analysis to be performed.

## RESULTS

*Batrachochytrium dendrobatidis* was detected on 30.3% ( $n = 519$ ) of the frogs we sampled (Table 1). However, the fungus was not detected on any frogs breeding in ephemeral waterbodies, and was detected on only a single terrestrial-breeding frog (Table 2). Frogs breeding in permanent waterbodies were significantly more likely to be infected than were frogs breeding in ephemeral or terrestrial habitats ( $\chi^2 = 62.3$ ;  $P < 0.0001$ , d.f. = 1), and frogs breeding in permanent streams were significantly more likely to be infected than were frogs breeding in permanent ponds ( $\chi^2 = 7.7$ ;  $P < 0.006$ , d.f. = 1).

The number of *B. dendrobatidis* zoospores detected on infected frogs ranged from 1 to 235,287 (geometric mean = 169; median = 183). There was no significant difference between the number of zoospores detected on permanent-stream and permanent-pond breeding frogs ( $t = -0.818$ ;  $P = 0.41$ ). The upper range of chytrid zoospores infecting permanent-stream breeders, however, was over an order of magnitude higher than that of permanent-pond breeders (Fig. 1).

We only detected *B. dendrobatidis* on a single terrestrial-breeding frog, an *A. darlingtoni* on whose swab 189 *B. dendrobatidis* zoospores were detected. We analysed this frog's swab a total of five times to confirm the diagnosis, and positive results were obtained each time. Furthermore, the number of zoospores detected on the sample was far higher than would have been



**Figure 1** Number of *Batrachochytrium dendrobatidis* zoospores detected on infected frogs from each breeding habitat. *B. dendrobatidis* was not detected on any ephemeral breeder.

Species	No. of positives	<i>n</i>	% Positive	Zoospores	Breeding habitat
<i>Litoria pearsoniana</i>	43	70	61.4	142	PS
<i>Adelotus brevis</i>	1	2	50.0	11030	PS
<i>Litoria wilcoxii</i>	65	161	40.4	280	PS
<i>Litoria chloris</i>	21	69	30.4	60	PS
<i>Litoria latopalmata</i>	1	5	20.0	484	PS
<i>Mixophyes iteratus</i>	0	1	0	–	PS
<i>Litoria fallax</i>	16	33	48.5	224	PP
<i>Litoria tyleri</i>	3	8	37.5	501	PP
<i>Limnodynastes peronii</i>	1	5	20.0	2	PP
<i>Litoria peronii</i>	3	17	17.6	19	PP
<i>Uperoleia fusca</i>	1	10	10.0	5	PP
<i>Litoria latopalmata</i>	1	21	4.8	7	PP
<i>Assa darlingtoni</i>	1	23	4.3	189	T
<i>Litoria fallax</i>	0	2	0	–	EP
<i>Litoria gracilentia</i>	0	37	0	–	EP
<i>Limnodynastes peronii</i>	0	1	0	–	EP
<i>Pseudophryne raveni</i>	0	10	0	–	EP
<i>Litoria chloris</i>	0	7	0	–	ES
<i>Litoria gracilentia</i>	0	37	0	–	ES
All frogs	157	519	30.3	169	

**Table 1** Distribution of *Batrachochytrium dendrobatidis* infection across species and breeding habitats. Zoospores = geometric mean number of *B. dendrobatidis* zoospores detected on infected frogs. PS = permanent stream; PP = permanent pond; ES = ephemeral stream; EP = ephemeral pond; T = terrestrial.

**Table 2** Prevalence and intensity of *Batrachochytrium dendrobatidis* infection as a function of frog breeding habitat. Zoospores = geometric mean number of *B. dendrobatidis* zoospores detected on infected frogs.

Breeding habitat	No. of positives	<i>n</i>	% Positive	Zoospores
Permanent stream	131	308	42.5	181
Permanent pond	25	94	26.6	114
Terrestrial	1	23	4.3	189
Ephemeral stream	0	44	0	–
Ephemeral pond	0	50	0	–

expected if the positive result were due to airborne contamination. As such, we have no reason to doubt the integrity of the result.

*Assa darlingtoni* was the only species sampled in 2006. To confirm that the low prevalence of *B. dendrobatidis* in the species was not due to some unexpected decrease in chytrid levels throughout the entire valley's frog community that year, we sampled 30 permanent-stream breeding *Litoria wilcoxii* 3 days after sampling the *A. darlingtoni* population (distance between the two populations = 4.5 km). Twelve *L. wilcoxii* were infected (two carried over 10,000 chytrid zoospores), confirming that the low infection prevalence in *A. darlingtoni* was not due to having sampled in a different year.

## DISCUSSION

The introduction of *B. dendrobatidis* to a naïve amphibian community can result in high rates of mortality and rapid population decline (Lips *et al.*, 2006; Rachowicz *et al.*, 2006; Schloegel *et al.*,

2006). Our results demonstrate that amphibians breeding in permanent waterbodies, and particularly in permanent streams, are at the highest risk of becoming infected with *B. dendrobatidis*. Consequently, they are also at the highest risk of chytridiomycosis-related population decline and extinction. Our findings strongly suggest that chytridiomycosis is one of the principle 'unidentified processes' (*sensu* Stuart *et al.*, 2004) responsible for the disappearance of stream-dwelling amphibian populations from protected areas throughout the world (e.g. Richards *et al.*, 1993; Drost & Fellers, 1996; La Marca *et al.*, 2005).

Though our study focused on a small area of Australia, and thus needs to be replicated in other geographical regions to ensure the generality of our findings, our results parallel those of field surveys conducted at times of mass amphibian mortalities. For example, Lips (1999) surveyed both stream and terrestrial transects in western Panama. The stream transects yielded 54 dead or dying amphibians (15% of total captures) of 10 species, while the terrestrial surveys yielded no dead or dying individuals. *Batrachochytrium dendrobatidis* was detected on all dead or dying individuals that underwent histological examination ( $n = 18$ ). Within 6 months, both the number of species and the number of individuals encountered along the streams decreased significantly (from 307 animals of 24 species to six animals of five species, with roughly equivalent search effort), while the number of species encountered along terrestrial transects remained the same ( $n = 7$ ). At another site in Panama, where *B. dendrobatidis* was detected on 315 of the 318 dead animals examined, 340 dead anurans were found along stream transects, but only six were found along terrestrial transects (Lips *et al.*, 2006). A significant decline in both amphibian density and species richness was apparent on stream transects, but no such decreases were evident for terrestrial transects.

The propensity of *B. dendrobatidis* to infect amphibians breeding in permanent streams is likely due in large part to the physiological limitations of the fungus. *Batrachochytrium dendrobatidis* can survive up to 7 weeks in sterile lake water (Johnson & Speare, 2003), but it cannot survive desiccation: 100% mortality takes place within 3 h of drying (Johnson *et al.*, 2003), and no resting stage has been identified (Longcore *et al.*, 1999), making its long-term persistence in ephemeral waterbodies unlikely. Though *B. dendrobatidis* has motile, flagellated zoospores (Longcore *et al.*, 1999; Berger *et al.*, 2005), they rarely swim more than 2 cm prior to encysting (Piotrowski *et al.*, 2004), suggesting that dissemination would be greatly assisted by flowing water. Furthermore, the fungus prefers cool temperatures (Longcore *et al.*, 1999; Berger *et al.*, 2004; Piotrowski *et al.*, 2004; Ouellet *et al.*, 2005; Kriger & Hero, 2007), and thus should grow better in streams than in ponds, as streams generally operate at lower temperatures than do ponds (ponds are exposed to more sunlight and do not carry water from cool, upland areas).

The habits and life histories of amphibians with aquatic larvae are also likely to be important factors explaining the differential infection levels between the ecological guilds. The larval stage of amphibians breeding in permanent waterbodies can last up to several years (Bury & Adams, 1999). As such, these tadpoles have ample opportunity to become infected with the waterborne zoospores of *B. dendrobatidis*. Furthermore, tadpoles serve as ideal reservoir hosts for *B. dendrobatidis* (Woodhams & Alford, 2005), and can transmit infection to uninfected adults (Rachowicz & Vredenburg, 2004). And whereas terrestrial breeders tend to be evenly distributed throughout the forest, amphibians breeding in permanent waterbodies often form dense aggregations at the breeding site, and thus are at increased risk of coming into contact with infected conspecifics. These aspects of *B. dendrobatidis* host–parasite ecology likely predispose the fungus towards infecting amphibians breeding in permanent streams, and make it unlikely that the fungus would infect fully terrestrial amphibians that bypass the aquatic tadpole stage.

However, our results and those of others (Waldman, 2001; Lips *et al.*, 2003b; Cummer *et al.*, 2005; Kriger & Hero, 2006) demonstrate that it is indeed possible for amphibians breeding away from permanent water to become infected with *B. dendrobatidis*. In the current study, infection was only detected on one such frog, a terrestrial-breeding *A. darlingtoni*. This is a cryptic species that spends the majority of its time under leaf litter, and is thus unlikely to make direct contact with sympatric frog species. The most plausible explanation for how this frog acquired chytrid infection is via contact with the infected shed skin of a sympatric frog species. *Litoria chloris*, a frog species on whom we often find *B. dendrobatidis*, was heard calling within 100 m of the infected *A. darlingtoni*. Juvenile and female *L. wilcoxii* (another species with high infection prevalence) can travel great distances from streams and thus serve as potential vectors as well. Regardless, the low infection prevalence we found in terrestrial and ephemeral breeding frogs, and the rarity with which infections are reported in these species, suggests that they are at low risk of acquiring chytridiomycosis.

The difference in chytrid infection levels between frogs breeding in permanent streams and those breeding in permanent ponds was less well defined. While permanent-stream breeding frogs had significantly higher infection prevalence than did permanent-pond breeding frogs, we found no significant difference in infection intensity between the two guilds. Though both permanent-pond and permanent-stream breeding species are clearly susceptible to *B. dendrobatidis* infections, our finding of higher prevalence in stream frogs is consistent with the worldwide pattern of chytrid-related mass mortality events, most of which have been reported from streams (e.g. Berger *et al.*, 1998; Lips *et al.*, 2006) and chains-of-ponds (low-energy stream systems of intermittent or discontinuous flow that contain deep, permanent ponds; e.g. Bosch *et al.*, 2001; Muths *et al.*, 2003; Rachowicz *et al.*, 2006), with few reports having emerged of die-offs at isolated ponds.

The results of our survey have important implications for amphibian research and conservation. First, many surveys are currently being planned to determine the presence or absence of *B. dendrobatidis* across large geographical areas (e.g. Speare *et al.*, 2005). By focusing their sampling on the ecological guilds most likely to carry chytrid infections, researchers conducting these surveys can reduce the number of samples required (DiGiacomo & Koepsell, 1986) and the likelihood of falsely declaring a site chytrid-free. As such, we recommend focusing these disease surveys on permanent-stream breeding amphibian populations. Second, the shortage of long-term population trend data is a well-known problem in amphibian research (Pechmann *et al.*, 1991; Skelly *et al.*, 2003), and currently the threat status and population trends of approximately 23% of the world's amphibian species cannot be assessed due to insufficient population data (Stuart *et al.*, 2004). Thus, many re-surveys of historical localities have taken place in recent years (Drost & Fellers, 1996; Lips *et al.*, 2004; Bustamante *et al.*, 2005; La Marca *et al.*, 2005), and many new monitoring programs can be expected to commence in the near future. In protected areas, where the negative effects of habitat destruction, over-harvesting, and invasive species can be assumed to be minimal, we suggest that future monitoring of amphibian populations give highest priority to permanent-stream breeding populations, as they are at the highest risk of chytridiomycosis-related decline. Finally, there is currently no known method for eradicating *B. dendrobatidis* from areas in which it has become established (DEH, 2005), and it is inevitable that the fungus will eventually invade at least some of the areas that are currently chytrid-free (Lips *et al.*, 2006). As such, it is important to mitigate all other threats to high-risk species, such as those breeding in permanent streams. Actions should include purchasing and preserving wide riparian corridors (and enforcing their protection) and severely restricting the commercial trade of highly sought-after stream-breeding species (i.e. dendrobatids). Lest these actions prove insufficient to combat the extirpation of wild populations, captive husbandry research should be focused on improving techniques used to breed amphibian species associated with permanent streams.

In conclusion, we have demonstrated drastic variation in the prevalence of *B. dendrobatidis* infection across sympatric species

that exhibit differing degrees of association with aquatic habitats, and we have identified permanent waterbodies, and permanent streams in particular, as the breeding habitats most likely to harbour significant *B. dendrobatidis* populations. The results of our study will aid amphibian researchers involved in disease surveys and population monitoring, and will enable wildlife managers to prioritize land management and enact appropriate trade restrictions. Furthermore, our study brings us a major step closer to resolving the puzzle of declining amphibian populations (Blaustein & Wake, 1995; Collins & Storfer, 2003; McCallum, 2005).

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