

Survivorship in Wild Frogs Infected with Chytridiomycosis

Kerry M. Kriger and Jean-Marc Hero

Centre for Innovative Conservation Strategies, Griffith University, PMB 50 Gold Coast Mail Centre, Southport, Queensland 9726, Australia

Abstract: Chytridiomycosis is an emerging infectious disease that has been implicated as the causative agent of many recent amphibian population declines and extinctions that have taken place in relatively pristine locations worldwide. While there exists a growing body of literature regarding the effect of the fungus on experimentally infected frogs, few studies have examined the effect of the fungus on apparently healthy wild frogs from nondeclining, infected populations. We examined the temporal pattern of chytrid infection in individually marked Stony Creek Frogs (*Litoria wilcoxii*) at a lowland site in southeast Queensland, Australia. We provide the first evidence that wild frogs are capable of both acquiring chytridiomycosis as adults, and also of clearing their infections entirely. Changes in disease status in individual frogs largely tracked changing climatic conditions, with infections tending to appear in cooler months and disappearing in warmer months. Though 27.2% of the adult frogs we sampled were infected at some point in the study, we found no evidence that chytridiomycosis was negatively affecting adult survivorship, suggesting either: (1) chytrid-induced mortality in this population is generally restricted to metamorphs and juveniles; (2) this population was not exposed to conditions which favored lethal disease outbreaks; or (3) this population has evolved sufficient resistance to the disease to persist relatively unaffected.

Key words: *Batrachochytrium dendrobatidis*, amphibian declines, chytridiomycosis, survivorship, chytrid, *Litoria wilcoxii*

INTRODUCTION

Amphibian population declines have become a major focus of conservation biology in recent decades, with up to one-third of amphibian species threatened with extinction (Stuart et al., 2004). Causal factors of these declines likely include habitat loss (Hero and Morrison, 2004), over-exploitation (Lannoo et al., 1994; Jensen and Camp, 2003), invasive species (Gillespie, 2001; Kats and Ferrer, 2003), climate change (Pounds, 2001; Williams et al., 2003;

Pounds et al., 2006), pollutants (Davidson et al., 2001; Hayes et al., 2002), and disease (Carey, 2000; Green et al., 2002). Most recently, the emerging infectious disease, chytridiomycosis, has been implicated as the cause of many declines that have taken place in protected areas throughout the world (Berger et al., 1998; Lips, 1999; Bosch et al., 2001; Bradley et al., 2002; Weldon and du Preez, 2004; La Marca et al., 2005). Chytridiomycosis is a cutaneous infection caused by *Batrachochytrium dendrobatidis*, a chytrid fungus which reproduces via waterborne zoospores, and parasitizes the mouthparts of larvae and the keratinized epidermis of post-metamorphic amphibians (Berger et al., 1998; Longcore et al., 1999; Marantelli et al., 2004).

A variety of outcomes are possible for amphibian populations infected with *Batrachochytrium dendrobatidis*, ranging from no impact to mass mortality and decline without recovery (Daszak et al., 1999; Daszak et al., 2003). Some infected populations of a species may decline, while other infected populations of the same species do not (McDonald and Alford, 1999), and declining species may coexist with nondeclining species at an infected site (Lips, 1999; Carey, 2000; Collins and Storfer, 2003). High variation in the population-level effects of chytridiomycosis is mirrored by a wide range of disease outcomes at the individual-level, as susceptibility of captive amphibians to chytridiomycosis has been shown to vary across species, age classes, and thermal conditions (Lamirande and Nichols, 2002; Davidson et al., 2003; Berger et al., 2004; Daszak et al., 2004). For instance, while *Rana catesbeiana* can survive inoculations of 10 million *B. dendrobatidis* zoospores per day for 31 days (Daszak et al., 2004), *Mixophyes fasciolatus* have been killed by as few as 100 zoospores (Berger et al., 1999). Not all experimentally infected *M. fasciolatus* die however, with those kept at 27°C significantly more likely to survive than those kept at 17°C or 23°C (Berger et al., 2004). In a laboratory experiment, Lamirande and Nichols (2002) infected *Dendrobates tinctorius* of various age classes: whereas all the larvae and recent metamorphs eventually died of chytridiomycosis, the juvenile and adult frogs not only survived, but cleared their infections.

Laboratory conditions are quite different from natural conditions. What is a sub-lethal infection in a laboratory setting could potentially be lethal under natural circumstances, or could reduce a wild animal's fitness to the point where it can be easily preyed upon, or be unable to acquire food resources (Quimby et al., 2005). Furthermore, it is unclear how many *B. dendrobatidis* zoospores wild animals are generally exposed to upon infection, and thus how realistic laboratory trials are. The results of experimental infections therefore cannot necessarily be extrapolated to wild animals (Parris and Cornelius, 2004). For instance, while chytridiomycosis is invariably fatal to *Rana muscosa* in laboratory trials, mathematical modeling suggests that at least some individuals must be able to survive infections in the wild (Briggs et al., 2005). This conflicting result highlights our need for empirically derived data on both the individual and the population-level effects of chytridiomycosis in wild frogs.

While it is clear that *B. dendrobatidis* can cause mass mortality and population declines in naive amphibian populations (Lips et al., 2006), there is little known

regarding the effects of the fungus on amphibian populations in which it has become established. To date, there has only been one published study (Retallick et al., 2004) that has used empirical data to examine survivorship in an infected population of apparently healthy, wild frogs. In that study, *Taudactylus eungellensis* survived up to 1089 days after initial diagnosis with *B. dendrobatidis*. However, due to the sampling method used, the authors were unable to determine whether the surviving frogs still carried chytrid infections, or whether they had cleared their infections completely. It was also unknown whether the frogs in the study had acquired their infections as adults or as tadpoles, the life stage where most anurans are likely to be exposed to the waterborne zoospores of *B. dendrobatidis*. Further, while recapture rates of infected frogs in that study (Retallick et al., 2004) were less than the recapture rates of uninfected frogs, and one of their statistical analyses suggested a significant decrease in survivorship of infected individuals, the authors concluded that there was no consistent evidence that survival differed between infected and uninfected frogs, leaving the issue of survivorship unanswered.

In this study we use real-time polymerase chain reaction (qPCR) to detect and quantify the number of *B. dendrobatidis* zoospores found on wild, individually marked Stony Creek Frogs (*Litoria wilcoxii*). By repeatedly sampling recaptured frogs over 1 year, we were able to construct infection histories for individual frogs and thereby shed light on some of the important issues left unresolved by past studies. Specifically, we aimed to determine: (1) if wild frogs were capable of clearing their chytrid infections; (2) if wild frogs acquire chytridiomycosis as adults; and (3) if chytridiomycosis affects survivorship in a nondeclining amphibian population.

METHODS

Study Species

The Stony Creek Frog, *Litoria wilcoxii* (Anura: Hylidae; formerly *Litoria lesueuri*), is one of the most common frogs found along streams in eastern Australia. The species is nocturnal, sexually dichromatic (breeding males have a conspicuous yellow coloration), and sexually dimorphic (females are often four times heavier and 50% longer than males). Breeding males aggregate along rocky sections of stream at night in the spring and summer months. The species is most common along streams running through

open forest or farmland but is occasionally sighted in closed rainforest. There is little evidence of any population decline in *Litoria wilcoxii* in recent decades, even in locations where sympatric species have disappeared (Gillespie and Hines, 1999). It has a very large geographical distribution, covering much of the eastern seaboard of Australia (Donnellan and Mahony, 2004), and has been implicated as a reservoir host for *B. dendrobatidis* that may serve to maintain and spread the infection throughout the region (Retallick et al., 2004).

Study Site

Frogs were sampled along a 1 km stretch of the Nerang River (28.177°S, 153.228°E, 155 m altitude), in Numinbah Valley, southeast Queensland, Australia. The site is on private property and encompasses both forested areas and cleared areas where cattle graze and utilize the riparian zone. The site supports a diverse amphibian assemblage that includes *Adelotus brevis*, *Limnodynastes peronii*, *Litoria peronii*, *L. pearsoniana*, *L. latopalmata*, *L. gracilentia*, *Mixophyes fasciolatus*, the endangered *M. iteratus*, and the introduced *Bufo marinus*.

Field Methods

Sampling took place at approximately 6-week intervals between February 3, 2005 and January 14, 2006. *Litoria wilcoxii* were caught using clean, unused 20 × 25 cm plastic bags. We sampled each frog for chytridiomycosis by firmly running a cotton swab (Kriger et al., 2006a) 10 times over each of the following locations: (1) the frog's dorsal surface; (2) each of the frog's sides, from groin to armpit; (3) the ventral surface; and (4) the undersides of each thigh. 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, and were frozen at -20°C upon return from the field (within 8 hours of sampling). All frogs were handled within the plastic bag or with unused nonpowdered latex gloves so as to prevent disease transmission between animals.

Frogs were individually marked by toe-clipping at the penultimate joint, using a numeric system (Hero, 1989). Scissors were sterilized in 70% ethanol and flamed prior to use on each frog, so as to (1) minimize the chance of disease transfer between frogs, and (2) destroy any residual DNA that could compromise the usefulness of the toes in future genetic tests. Toes were stored in 70% ethanol in individual vials, and are available from the corresponding

author for use in future studies (i.e., genetic, disease, or skeletochronology research). We used a Thermochron iButton DS1921G temperature logger (Dallas Semiconductor, sourced from Alfatek, Bayswater, Victoria, Australia) to record air temperature at the site every 90 minutes. The temperature logger was placed at ground level in a shaded area ~10 m from the river's edge, so as to best approximate the temperature in a frog's diurnal refugium.

Laboratory Methods

Swabs were analyzed for the presence of *Batrachochytrium dendrobatidis* using established quantitative (real-time) PCR techniques (Boyle et al., 2004), and employing the changes described by Kriger et al. (2006b).

Data Analysis

We assumed that if chytridiomycosis was negatively affecting survivorship, the recapture rate for infected frogs would be significantly less than that of uninfected frogs. We performed a Chi-squared test, with animals separated into four groups as follows: (1) infected frogs that were eventually recaptured; (2) infected frogs that were never recaptured; (3) uninfected frogs that were eventually recaptured; and (4) uninfected frogs that were never recaptured. Two frogs whose infection status changed from negative to positive prior to final recapture were placed in the first group. Placing these frogs in the third group would not have affected the qualitative results of the analysis. Frogs initially caught on the final sampling date (January 14, 2006) were not included in the analysis because they were not available for recapture.

Quantification of chytrid zoospores on infected frogs is presented as the mean value of *B. dendrobatidis* genome equivalents detected in the three replicates of the triplicate PCR analysis. We use this number as an index of the severity of a frog's infection (parasite load). Because the number of zoospores detected varied over five orders of magnitude, data were log transformed prior to statistical analyses. An independent *t*-test was used to determine if there was a significant difference between the number of *B. dendrobatidis* zoospores found on positive frogs that were eventually recaptured and those that were never recaptured.

Qualitative results did not differ whether we examined adult male frogs, all adult frogs, or adults and juveniles combined. However, because the response of juvenile frogs to chytrid infections can differ from that of adults, as mentioned previously, and because sample sizes in juveniles were low, we restricted all statistical analyses to adult frogs.

Table 1. Prevalence of *Batrachochytrium dendrobatidis* infection in *Litoria wilcoxii*, number of frogs marked, and number of recaptured frogs with various infection histories^a

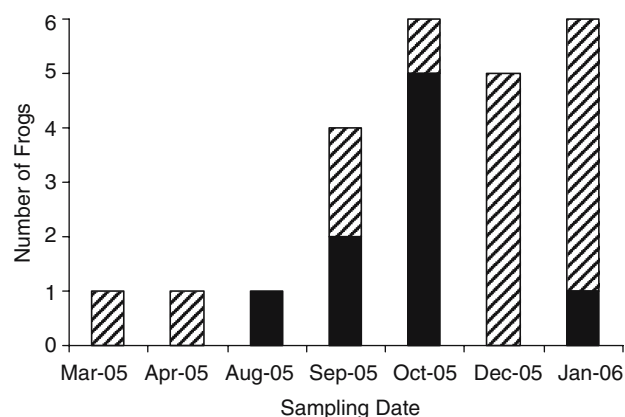
	Infected (%)	Marked	N → N	N → P	P → N	P → P	P → N → P	N → P → N
Males	32.5	123	15	2	5	0	0	2
Females	0	24	2	0	0	0	0	0
Juveniles	10.0	10	0	0	0	0	0	0
Total	26.1	157	17	2	5	0	0	2

^aN = negative, P = positive. First letter represents infection status upon initial capture, final letter represents infection status upon final capture, and middle letter represents an intermediate capture that yielded a different result than did the initial and final captures.

RESULTS

A total of 157 *Litoria wilcoxii* were marked during the study, of which 26 individual frogs were recaptured. *Batrachochytrium dendrobatidis* was detected on 32.5% of the adult males ($n = 123$), none of the adult females ($n = 24$), and 10% of the juvenile frogs ($n = 10$). No frog tested positive on multiple occasions, but seven adult males that tested positive eventually tested negative (Table 1). Two of these frogs tested negative on two separate occasions after initial positive diagnosis, and one frog tested negative on three separate occasions after initial positive diagnosis, making it unlikely that the frogs were actually infected during subsequent recaptures and that we simply failed to detect the fungus. Four adult males that were negative upon initial capture tested positive upon recapture. The temporal pattern of infection in frogs whose disease status changed during the course of the study generally tracked the seasons, with infections present mainly in the cooler months and rare in the warmer months (Fig. 1; Table 2).

There was no evidence that infection by *B. dendrobatidis* affected survivorship of adult *L. wilcoxii* ($\chi^2_{1,0.05} < 0.01$; $P = 0.97$), and recapture rates of infected and uninfected frogs were virtually identical (Table 3). Nevertheless, our overall recapture rate was low (18%) and only 26 total frogs were recaptured, so we may not have had sufficient power to detect an effect if one did indeed exist. There was no significant difference between the number of zoospores found on infected frogs that were eventually recaptured and infected frogs that were never recaptured (t -value = -0.28 ; $df = 38$; $P = 0.78$; Fig. 2), though there were no recaptures of frogs on whom more than 4421 chytrid zoospores were detected ($n = 8$). The mean length of time elapsed between a frog being diagnosed positive for *B. dendrobatidis* and its final recapture was 103 days (range: 54–132; SD: 27), which well exceeds

**Figure 1.** Seasonal distribution of *Batrachochytrium dendrobatidis* infection for the nine recaptured *Litoria wilcoxii* whose infection status changed during the course of the study (diagonal fill: negative PCR results; black fill: positive PCR results). The nine frogs were sampled a total of 24 times.**Table 2.** Temporal pattern of *Batrachochytrium dendrobatidis* infection for the nine recaptured adult male *Litoria wilcoxii* whose infection status changed during the course of the study^a

Frog	Mar-05	Apr-05	Aug-05	Sep-05	Oct-05	Dec-05	Jan-06
1	N			235,287			
2		N	2112	N		N	
3					552	N	
4				185	N	N	N
5					60		N
6					642		N
7					4421		N
8				N	46	N	N
9						N	180

^aNegative PCR results denoted by "N," positive PCR results by the number of *B. dendrobatidis* zoospores detected on the infected frog.

the approximately 30-day period in which experimentally infected frogs often die of lethal chytridiomycosis (Nichols

Table 3. Survival of infected and uninfected adult *L. wilcoxii*

Infection status	Marked	Recaptured (proportion)
Positive	40	7 (17.5%)
Negative	107	19 (17.8%)

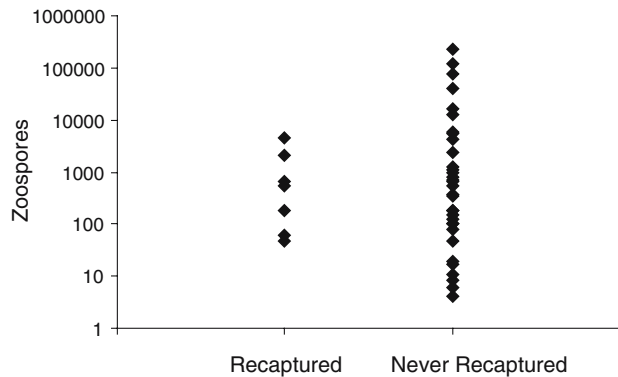


Figure 2. Number of *Batrachochytrium dendrobatidis* zoospores detected on infected *Litoria wilcoxii* that were eventually recaptured ($n = 7$), and those that were never recaptured ($n = 33$). All frogs in the former group cleared their infections by final recapture.

et al., 2001; Daszak et al., 2004). *Batrachochytrium dendrobatidis* was detected in all three wells of every positive frog's triplicate PCR analysis, making it unlikely that positive results were due to contamination.

DISCUSSION

While infection by *Batrachochytrium dendrobatidis* often leads to death in experimentally infected frogs (Berger et al., 1999; Briggs et al., 2005) and in wild frogs from naive populations with no prior exposure to the fungus (Lips et al., 2006), several laboratory trials have shown that some species are capable not only of surviving but also of clearing their infections completely (Lamirande and Nichols, 2002; Davidson et al., 2003). We have demonstrated that wild frogs are also capable of surviving and clearing their chytrid infections. The ability to overcome a potentially lethal infection confers a selective advantage on an individual, and such a trait is likely to be rapidly acquired after the initial introduction of an exotic pathogen to a population (May and Anderson, 1983). As *B. dendrobatidis* has been present in southeast Queensland for at least 25 years (Speare and Berger, 2005), it is not surprising that a highly

abundant, widespread frog species such as *L. wilcoxii* has at least some immunity to chytrid infections, and that while *L. wilcoxii* can acquire chytridiomycosis as adults, the disease does not appear to significantly reduce adult survivorship in the frog population we studied.

We hesitate to conclude, however, that *B. dendrobatidis* does not reduce adult survivorship in many amphibian populations, even long after it has become established at a site. A variety of factors can promote lethal disease outbreak in an amphibian population currently experiencing only aclinical infections, including anthropogenic stressors that serve to decrease immune response (Blaustein and Kiesecker, 2002), and changing weather patterns that favor the parasite (Pounds et al., 2006). Amphibian populations living in other locations (e.g., higher altitudes or latitudes further from the equator) may experience environmental conditions under which they are unable to combat infections. Also, amphibian species vary in their innate abilities to combat infections (Rollins-Smith and Conlon, 2005), and it is possible that certain species may never acquire immunity to chytridiomycosis. This could explain the inability of several of Queensland's endangered frog species to recolonize the upland habitats from which they were extirpated decades ago (i.e., *Litoria nannotis*, *L. rheocola*, *Nyctimistes dayi*, and *Taudactylus eungellensis*).

All seven frogs that cleared their infections first tested positive in August, September, or October, the time of year when infection prevalence in this population peaks (Kriger and Hero, 2006). All frogs eventually tested negative by December, when the average daily maximum air temperatures (measured in the shade) had risen from 20.7°C to 25.2°C. Maximum temperatures along unshaded sections of the stream were likely several degrees higher, and during the latter period may have regularly exceeded the thermal limits of *B. dendrobatidis* (29°C in vitro; Longcore et al., 1999). As climatic conditions ceased to be suitable for the survival of the fungus, the balance was likely tipped in favor of the infected frogs, whose skin peptides are capable of inhibiting low densities of *B. dendrobatidis* zoospores (Woodhams et al., 2005).

The high prevalence of chytridiomycosis in this *L. wilcoxii* population, along with the apparently low number of lethal infections and the wide geographical area in which the species is found, supports the conclusions of Retallick et al. (2004), who implicated *L. wilcoxii* as both a vector and a reservoir species for the disease. *Litoria wilcoxii* inhabits many of the dry lowland sections that separate the major rainforests of Queensland, and the species may

therefore serve as a “bridge” that can transfer disease to rainforest frog communities in disparate locations.

In summary, we have demonstrated that wild frogs can acquire chytridiomycosis as adults, and that they are capable of clearing their chytrid infections entirely. Both of these changes in disease status largely track changing climatic conditions, with infections tending to appear in cooler months, and disappear in warmer months. Chytridiomycosis does not always lead to rapid death in infected *L. wilcoxii*, and did not appear to significantly affect adult survivorship in our study population. These results will be valuable to epidemiologists and amphibian researchers interested in disease dynamics in natural systems.

ACKNOWLEDGMENTS

We thank Peter and David Lyons for allowing us to conduct fieldwork on their property, and the many volunteers who assisted with fieldwork. We also thank two anonymous reviewers for providing valuable comments on an earlier draft of this manuscript. The Heart Foundation Research Centre graciously allowed access to PCR equipment. K.M.K. was partially supported by the Griffith University School of Environmental and Applied Sciences.

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